Vol. 10. No. 3. SEPTEMBER, 1955

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LONDON
BRITISH MEDICAL ASSOCIATION
TAVISTOCK SQUARE, W.C.1

YEARLY SUBSCRIPTION (4 NUMBERS) £4 4s. U.S.A. $15.50 SINGLE NUMBERS £1 5s.

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Brit. J. Pharmacol. (1955), **10**, 265.

ACTIONS OF SUCCINYLCHOLINE CHLORIDE ON THE CIRCULATION

BY

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From the Department of Pharmacology, Laboratorios Galien, Montevideo, Uruguay

(RECEIVED JANUARY 22, 1954)

The neuromuscular blocking properties of succinylcholine are well known, but reports on the action on the heart rate and blood pressure are more contradictory. Thesleff (1952) states that Bovet, Bovet-Nitti, Guarino, Longo and Fusco (1951) reported that succinylcholine iodide had both a muscarinic and a nicotinic effect on the blood pressure, but could not himself observe a fall in blood pressure of a muscarinic type in his own experiments. Somers (1953) also states that succinylcholine has only a nicotinic action. However, Hunt and Taveau (1911) reported that the injection of succinylcholine in a majority of intact animals caused a slowing of the heart rate which was not seen in atropinized or vagotomized animals ; in the latter, succinylcholine caused a prolonged hypertension. Exceptionally, they observed a hypotension like that caused by muscarine. They also found that succinylcholine could oppose the hypotensive effects of other muscarinic substances. It therefore seemed desirable to reinvestigate the effects of succinylcholine upon the circulation in view of these discrepancies in the literature.

Thesleff (1952) concluded that the increase in blood pressure seen with relatively high doses was not due to stimulation of the carotid sinus or carotid body, and that it was much diminished after blocking the ganglia with hexamethonium or tetraethylammonium. The latter observation has been corroborated by Vidal Beretervide (unpublished observations). Thesleff explained the rise in blood pressure after giving succinylcholine by stimulation of autonomic ganglia and mobilization of adrenaline.

However, in his experiments ganglion blocking substances did not entirely prevent a rise in blood pressure. After dibenamine there was an inversion of the blood pressure response followed by a slight rise in pressure ; a direct vasoconstrictor action of succinylcholine could not therefore be excluded. Experiments have therefore been performed to analyse the mechanism of action of succinylcholine in causing a rise of blood pressure. Most authors (Bovet *et al.*, 1951 ; Glick, 1941 ; Ginzel, Klupp, and Werner, 1951c ; Evans, Gray, Lehmann, and Silk, 1952) have found that succinylcholine is decomposed by pseudo cholinesterase and by true cholinesterase, whereas Bovet *et al.* state that succinylcholine inhibits pseudo cholinesterase but not true cholinesterase. Evans *et al.* found that succinylcholine was not destroyed by true cholinesterase but was metabolized by the pseudo cholinesterase of serum, though at a slower rate than ACh ; succinylcholine was found to be a competitive inhibitor of ACh hydrolysis by both esterases, the true esterase being the one more strongly inhibited. Finally Evans *et al.* (1952) say: "It is reasonable to assume that succinylcholine acts by inhibiting the true acetylcholine esterase at the neuromuscular junction, and that the removal of the drug by the pseudo cholinesterase of the serum normally curtails its effect." As support for this interpretation they recall that the longest response seen so far is that reported by Harper (1952) when neostigmine was given. If the hypothesis of Evans *et al.* is true, then the accumulation of ACh is the cause of the neuromuscular block when succinylcholine is administered. It therefore seemed desirable to determine whether succinylcholine potentiates ACh *in vivo* by inhibiting cholinesterase.

METHODS

Forty dogs and forty-five rabbits were used, either unanaesthetized or anaesthetized with pentobarbitone sodium (25–30 mg./kg.) or chloralose (0.1 g./kg.) intravenously. Drugs were injected intravenously. In conscious animals sufficient succinylcholine chloride was used to paralyse the respiratory muscles completely (0.12 mg./kg. for dogs ; 0.3–0.4 mg./kg. for rabbits). In anaesthetized animals the doses used were about 50 to 100 times the paralysing doses. Artificial respiration was always used. Blood pressure was recorded from the femoral artery with a mercury manometer.

Electrocardiograms, using classical or precordial leads, with hypodermic needles as electrodes, were recorded with a direct writing apparatus. Atropine sulphate was used in a dose of 2 mg./kg.; ACh was given in doses of 0.1–0.5 mg./kg.

The succinylcholine chloride used was synthesized in Laboratorios Galien. "Pendiomid" (azamethonium, A.N.) and "Regitine" (phentolamine, A.N.) were obtained from the Ciba Laboratories.

Results

Conscious Animals

The intravenous injection of succinylcholine causes an immediate and profound bradycardia at the onset of paralysis. The heart rate falls from 250 to 50/min. (rabbit) or from 100 to 30/min. (dog) with slight variations of frequency superimposed (Fig. 1). Artificial respiration causes the heart rate to return to normal in 10–15 sec., and it falls to the previous value 15–20 sec. after ceasing artificial respiration. In rabbits, inflation of the lungs with nitrogen gives precisely the same results. This bradycardia is not seen if the animal is anaesthetized. Fig. 2 shows the effect of injecting pentobarbitone in conscious animals which had previously been injected with succinylcholine, but had not been treated by artificial respiration; the bradycardia ceased and the heart resumed its normal rhythm. A similar result was obtained by injecting 1.5–2 ml. of 10% procaine intracisternally in rabbits. Severing or anaesthetizing the vagi, or giving atropine, also prevents the bradycardia. In anaesthetized animals it was confirmed that vagal excitability was not modified even after administration of very large doses of succinylcholine. When such large doses were given, a profuse salivation and augmented tear secretion were observed both in dogs and rabbits, as Bovet *et al.* (1951) and Ginzel *et al.* (1951) have reported.

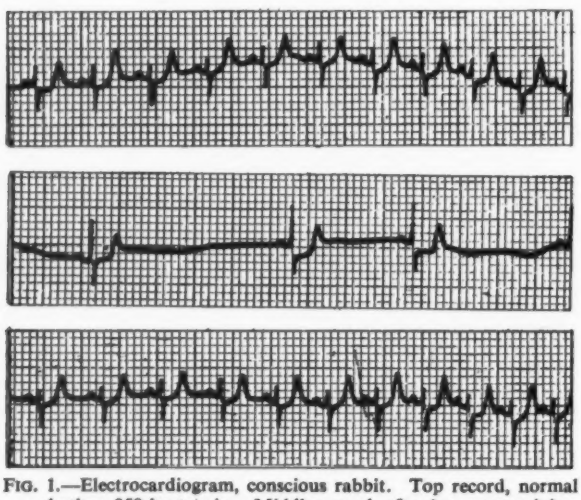

FIG. 1.—Electrocardiogram, conscious rabbit. Top record, normal rhythm, 250 beats/min. Middle record, after intravenous injection of 1 mg. succinylcholine chloride without artificial respiration, 60 beats/min. with sinus arrhythmia. Bottom record, 10 sec. after artificial respiration was begun; 250 beats/min.

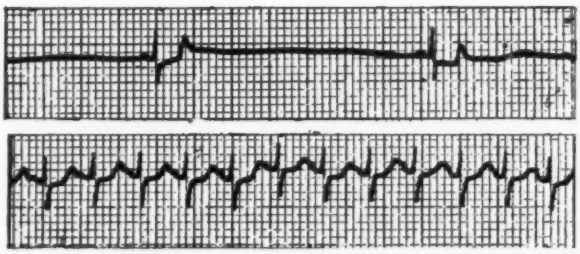

FIG. 2.—Electrocardiogram, conscious rabbit. Upper record, after the injection of succinylcholine without artificial respiration, 40 beats min. Lower record, a few seconds after intravenous injection of pentobarbitone; the rate has returned to 215 beats/min.

The femoral arterial blood pressure was recorded in rabbits under local anaesthesia (1–2% procaine). After an injection of succinylcholine the blood pressure was not altered for 60–90 sec. after the beginning of the bradycardia. It then began to fall, apparently by diminution of the minute-volume, but 10–15 sec. after artificial respiration was renewed the blood pressure rose rapidly to its previous level.

Provided that artificial respiration was maintained, large doses of succinylcholine caused big rises in blood pressure in conscious rabbits (Fig. 3).

Anaesthetized Animals

Large doses of succinylcholine chloride always caused a rise of blood pressure in dogs, rabbits, and cats as Thesleff (1952), Bovet *et al.* (1951, 1952), and Bourne (1952) have found. Exceptionally, an initial slight and very transient fall of blood pressure preceded the rise in dogs. Bilateral adrenalectomy or ligature of the suprarenal vessels in dogs diminished the rise of blood pressure caused by succinylcholine chloride but did not abolish it (Figs. 4 and 5). Hexamethonium chloride or "Pendiomid" in doses of 5–10 mg./kg. completely abolished the hypertensive response; there was no residual hypertension such as that seen by Thesleff (1952). In the same way yohimbine and phentolamine abolished the rise of blood pressure but did not reverse it.

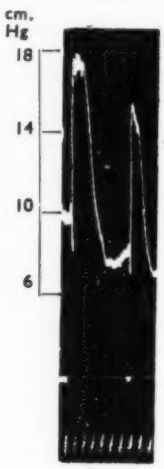

FIG. 3.—Conscious rabbit. Record of blood pressure from femoral artery. Pressor responses to 75 and to 50 mg. of succinylcholine i.v. Time, min.

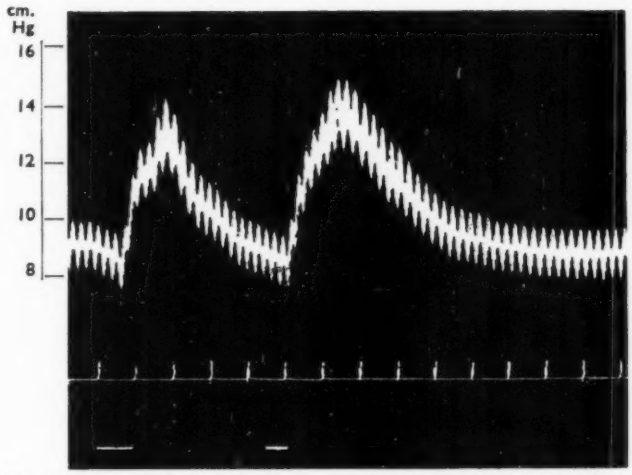

FIG. 4.—Adrenalectomized dog under pentobarbitone anaesthesia. Succinylcholine 50 mg. (at 1st signal) and 60 mg. (at 2nd signal) i.v. causes a rise in blood pressure. Time, 15 sec.

In dogs given 2 mg./kg. atropine sulphate the rise of blood pressure caused by the injection of ACh was considerably potentiated by administration of succinylcholine (Figs. 6 and 7). Not only was the hypertension caused by ACh much greater but it was of longer duration. In some such experiments subsequent injections of succinylcholine after administration of ACh caused smaller hypertensive responses, but the effect of ACh was always clearly increased. Succinylcholine also potentiated, though to a much smaller degree, the hypertensive effects of benzoylcholine in some experiments but did not potentiate the rise of blood pressure caused by nicotine or carbaminoylcholine. Nicotine actually blocked the effect of succinylcholine on the ganglia (Fig. 8). In anaesthetized dogs, which had not received atropine, succinylcholine did not reduce or abolish the fall in blood pressure caused by administration of ACh as Hunt and Taveau (1911) found, but on the contrary increased the fall of blood pressure. This was best demonstrated by injecting ACh during a constant intravenous infusion of succinylcholine, 0.1% w/v in normal saline, at a rate of 3–4 mg./min. (Fig. 9) or by injecting ACh at the peak of the hypertension caused by previous injections of succinylcholine. In this last instance, not only was the fall of pressure greater, but it lasted 8 to 10 min. instead of a few seconds.

DISCUSSION

In conscious animals succinylcholine chloride seems to act as a stimulant to the vagus centre, for bradycardia is only observed when the nervous centres are neither anaesthetized nor destroyed. Cutting the vagi or injecting atropine also abolishes the bradycardia. The bradycardia does not appear to be due to anoxia although artificial respiration abolishes it. Thus anoxia usually first causes tachycardia, and the modifications in the electrocardiogram due to anoxia—as, for instance, the depression of the S–T segment—did not appear in the tracings when bradycardia occurred. Moreover, the rapid disappearance of bradycardia with artificial respiration, and its disappearance on inflation of the lungs with nitrogen, also demonstrate that anoxia is not the determining factor. Bradycardia also does not occur in the anaesthetized animal, or in an animal whose bulbar centres have been paralysed by procaine and which is without artificial ventilation. These observations suggest that it is distension of the lungs, and not modification of the oxygen content of the blood, which causes the abolition of bradycardia by artificial ventilation. Though reports of the action of

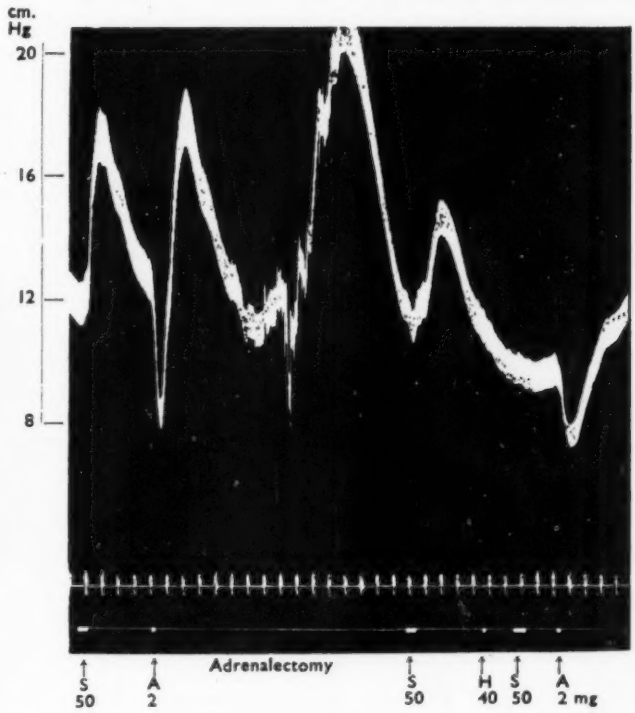

FIG. 5.—Dog, pentobarbitone anaesthesia, atropine sulphate, 1 mg./kg Injections of succinylcholine chloride (S) 50 mg. and acetylcholine (A) 2 mg. cause a rise of blood pressure. Adrenalectomy (giving rise to a hypertension during manipulations of adrenals). After adrenalectomy, succinylcholine chloride 50 mg. still causes a rise in blood pressure. After hexamethonium (H) 5 mg./kg., succinylcholine 50 mg. no more provokes a hypertension, and acetylcholine 2 mg. causes only a hypotension. Time, min.

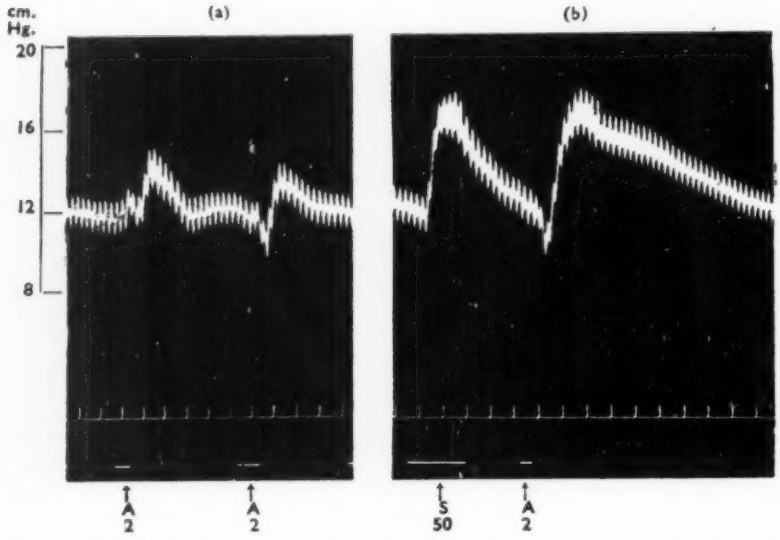

FIG. 6.—Dog, pentobarbitone anaesthesia, atropine sulphate 2 mg./kg. (a) Injections of acetylcholine chloride (A) 2 mg. (b) Injection of succinylcholine chloride (S) 50 mg. followed by acetylcholine chloride 2 mg. Succinylcholine potentiates the hypertension caused by acetylcholine. Time, 15 sec.

succinylcholine in conscious man (Thesleff, 1952; Mayrhoffer, 1952) do not mention cardiovascular changes, it seems advisable not to use succinylcholine in conscious man without previous administration of atropine, until it has been established that there is not a species difference.

Administration of large doses of succinylcholine to anaesthetized animals causes a rise of blood pressure by excitation of sympathetic ganglia and the adrenal glands. The part played by the adrenals is important in dogs; but a rise of blood pressure still occurs in their absence, and is probably due to direct stimulation of the ganglia.

Appropriate doses of ganglionic-blocking drugs abolish the rise of blood pressure. Administration of dibenamine and phentolamine also abolishes the hypertension, which excludes a direct vasoconstrictor action by succinylcholine. Succinylcholine also does not modify the pressor actions of adrenaline or noradrenaline.

The evidence suggests that succinylcholine does not act in competition with acetylcholine. In dogs it potentiates the muscarinic effects of ACh and it also increases its nicotinic action. Since succinylcholine itself has no vasodilator action, potentiation of the muscarinic action of ACh suggests that it may inhibit cholinesterase *in vivo*.

The small potentiation of the rise of blood pressure caused by benzoylcholine, and the absence of potentiation of the pressor effects of carbaminoylcholine and nicotine, suggest that this effect is due to the inhibitory action of succinylcholine on true cholinesterase, as demonstrated by Evans *et al.* (1952).

That succinylcholine does not potentiate the actions of carbaminoylcholine and nicotine is attributed to the fact that these substances are not split by cholinesterases. The feeble potentiation of benzoylcholine is explained by the weak inhibition by succinylcholine of pseudo cholinesterase, which hydrolyses benzoylcholine. Since succinylcholine does not affect the pressor action of adrenaline or noradrenaline, the potentiation of the response to acetylcholine by the enhancement of the activity of liberated adrenaline or noradrenaline can be excluded.

SUMMARY

1. Injection of succinylcholine to conscious animals causes profound bradycardia by central vagal stimulation. The bradycardia can be abolished by cutting the vagi, by administering atropine, by general anaesthesia, and by intracisternal injection of procaine.

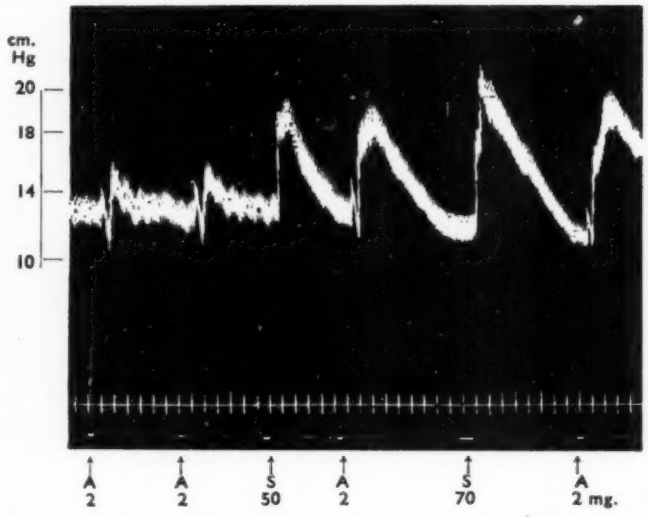

FIG. 7.—Dog, chloralose anaesthesia, atropine sulphate 2 mg./kg. Injections of acetylcholine chloride (A) 2 mg. before and after injections of succinylcholine chloride (S) 50 and 70 mg. Succinylcholine chloride potentiates the hypertension caused by injection of acetylcholine. Time, 30 sec.

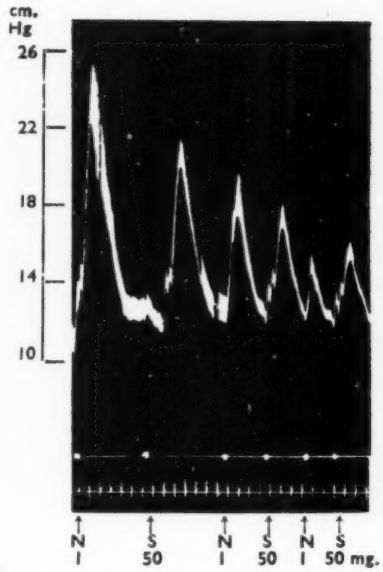

FIG. 8.—Dog, pentobarbitone anaesthesia. Blood pressure record. Nicotine sulphate (N) 1 mg. and succinylcholine chloride (S) 50 mg. are injected alternately. Hypertensions diminish progressively. Succinylcholine does not potentiate nicotine. Nicotine diminishes the pressor effect both of succinylcholine and of nicotine. Time, min.

2. This bradycardia can be abolished by artificial ventilation of the lungs with either air or nitrogen. It is suggested that this abolition is due to a reflex initiated by distension of the lungs.

3. Large doses of succinylcholine in anaesthetized or conscious animals under artificial ventilation cause a rise of blood pressure due to stimulation of sympathetic ganglia and the adrenal glands.

4. Succinylcholine potentiates the rise of blood pressure caused by injection of acetylcholine in atropinized animals, by inhibiting true cholinesterase. It does not act as a competitive substance for acetylcholine.

5. Succinylcholine has no direct actions on the blood vessels or on the heart.

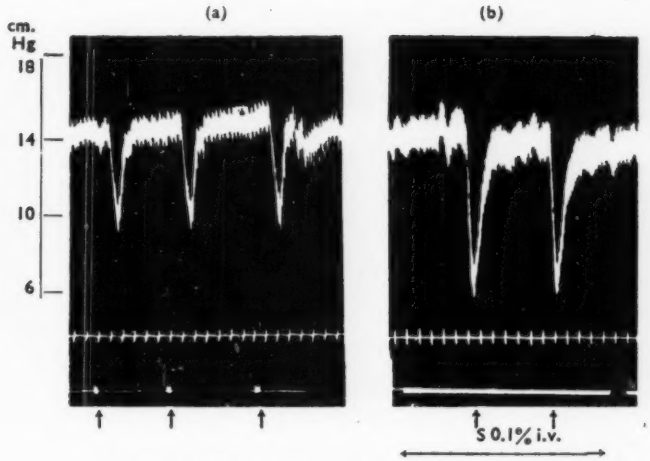

FIG. 9.—Dog, chloralose anaesthesia. At arrows, (a) 3 injections of acetylcholine chloride 50 μg. (b) 2 injections of acetylcholine chloride 50 μg., during a slow intravenous infusion of a solution of succinylcholine chloride (S) 0.1%. Succinylcholine potentiates the hypotension caused by injections of acetylcholine. Time, min.

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Brit. J. Pharmacol. (1955), **10**, 270.

SOME CHEMICAL AND PHYSICAL PROPERTIES ASSOCIATED WITH HISTAMINE ANTAGONISM

BY

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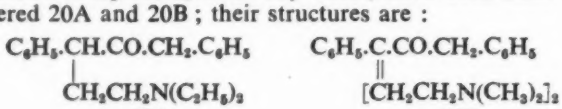
(RECEIVED MAY 10, 1954)

To study the relationships between chemical structure, physical properties and antihistamine activity, it is necessary to measure the relative potencies accurately, and to determine which compounds are competitive antagonists of histamine and which are merely non-competitive spasmolytics. The *p*A technique of Schild (1947) provides a method whereby both these objects may be achieved, and a relationship between antihistamine action and dissociation constant previously observed has been confirmed.

METHODS

The following series of compounds have been studied :

(1) The " Wilson " series, whose chemical structures have been recorded by Marshall, Ahmad, and Weston (1952). The numbers previously assigned to the compounds are also used in the present paper. Two further compounds, both oily bases, have been numbered 20A and 20B ; their structures are :

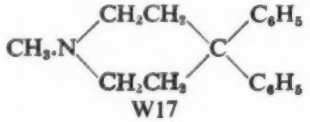


20A

20B

Potentiometric titration of 20B showed it to be chemically unstable, and it was not therefore included in the pharmacological investigation.

(2a) Methadone, and a series of related compounds which have been described by Walton, Ofner, and Thorp (1949) and by Ofner and Walton (1950) ; these are referred to in Table II by code numbers. Two compounds not referred to in the literature have the structures :



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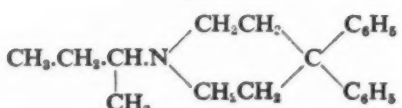

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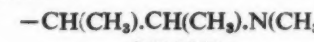
(2b) Phenadoxone, *iso*-phenadoxone, and five related compounds which are referred to in Table II by figures denoting the number of the compound in Table II of the paper by Dupré, Elks, Hems, Speyer, and Evans (1949).

(3) A series of proprietary antihistamine drugs.

(4) A series of phenothiazine derivatives. Structures of all but three of these have been described by Viaud (1954); these three have the following side-chain structures :



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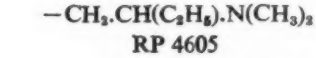

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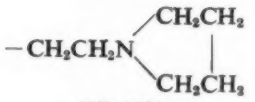
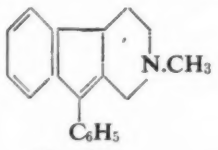


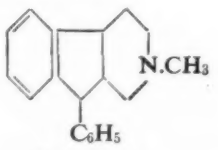
RP 4588

(5) A series of benzhydryl derivatives allied to chlorcyclizine (Table I).

(6) Two derivatives of phenindamine :



Nu-1326



Nu-1525

Measurement of Antihistamine Action

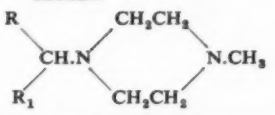
The *p*A values were determined by the method of Schild (1947) using an automatic organ bath in which guinea-pig ileum was suspended in oxygenated Tyrode solution at 36° C. Doses of histamine were added every 2 min. and contact with each concentration of antagonist was maintained for five cycles. The basic concentration of histamine was 0.02 μg./ml. Each pA_2 and pA_{10} value is the mean of the results on ileum from at least four guinea-pigs.

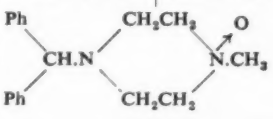
At first the *p*A values for the more potent antihistamines were discrepant because the basic compounds were adsorbed on to the glassware. This source of error has been described for botulinus

TABLE I

STRUCTURES OF BENZHYDRYL (CHLORCYCLIZINE) SERIES

General formula



Serial No.	R	R_1	Salt
A-1198 ..	Phenyl	Phenyl	HCl
A-2285 ..	*p*-Bromo-phenyl	,,	,,
A-2342 ..	*p*-Fluoro-phenyl	,,	,,
A-2688 ..	*p*-Methyl-phenyl	,,	,,
Chlorcyclizine	*p*-Chloro-phenyl	,,	,,
A-2824 ..	*m*-Chloro-phenyl	,,	,,
A-2917 ..	2-Thiophen	*p*-Chloro-phenyl	$2(COOH)_2$
A-4826 ..			,,

toxin by Bronfenbrenner and Schlesinger (1922), and for bases by Brodie, Udenfriend, and Baer (1947). Tests by experimental contamination of flasks and pipettes with chlorothen showed that adsorption on to glass surfaces could be eliminated by soaking the glassware in commercial conc. HNO_3 when not in use (to avoid the formation of surface films of substances such as detergents), and by using a minimum number of stages in preparing the high dilutions required for *p*A determinations.

Measurement of Dissociation Constants

Dissociation constants were calculated from data obtained by titrating fractional molar solutions of salts of basic compounds with standard KOH. Because of the small amounts of some compounds, 20 ml. portions of 0.0025M solutions were titrated with an equivalent volume of 0.1N-KOH. With monobasic compounds, 0.5 ml. 0.1N-KOH was added in ten 0.05 ml. portions, using an Agla micrometer syringe, clamped horizontally, and fitted with a glass attachment bent at right angles, with the fine tip 0.5 mm. above the surface of the solution. With dibasic compounds, 1.0 ml. 0.1N-KOH was required to complete the titration. After each addition, the *p*H of the solution was recorded by means of standard glass and calomel electrodes connected to a Cambridge *p*H meter. The titrations were carried out at 20° C. All solutions were prepared with distilled water boiled for 20 min. and stored in an aspirator fitted with a soda-lime absorption tube. The 0.1N-KOH was prepared from washed sticks of potassium hydroxide AR dissolved in boiled water. After standardization, the solution was adjusted to volume and stored in an aspirator of amber glass fitted with a soda-lime tube. The solution was re-standardized every 14 days, using the *p*H meter, the titration curve being checked against the theoretical curve in order to detect any conversion of hydroxide to carbonate (Fig. 1).

Where, as usually, the base was available as a salt of a strong acid such as sulphuric or hydrochloric, the salt was dissolved and titrated as such. Compounds supplied as the free base were dissolved in the stoichiometric amount of HCl before making up to volume with boiled water. Where the compound was supplied as the salt of a weak organic acid, such as citrate, maleate or phosphate, the weak acid affected the titration curve. Here it was necessary to convert the compound to a salt of a strong acid. Stoichiometric portions of the weak acid salt (usually sufficient to make 50 or 100 ml. of 0.0025M) were dissolved in water in a separating funnel, and the exact equivalent of 0.1N-KOH added to liberate the free base. The free base was transferred to ethereal solution by shaking with two successive 30 ml. portions of ether. The mixed ether extract was then re-extracted with an exact equivalent of 0.1N-HCl, in three successive portions. The mixed acid extract was heated on a water bath to drive out dissolved ether, and made up to volume with boiled water. Aliquots of 20 ml. of the hydrochloride were then titrated with 0.1N-KOH.

Where the free base precipitated during titration, it was necessary to carry out the titration in alcoholic solution. In this wide range of compounds it was not practicable to apply a constant alcohol correction of +0.5 *p*H unit as used by Albert, Rubbo, Goldacre, Davey, and Stone (1945) for a series of acridines, since the slopes of the pK_a – % alcohol curves differed for

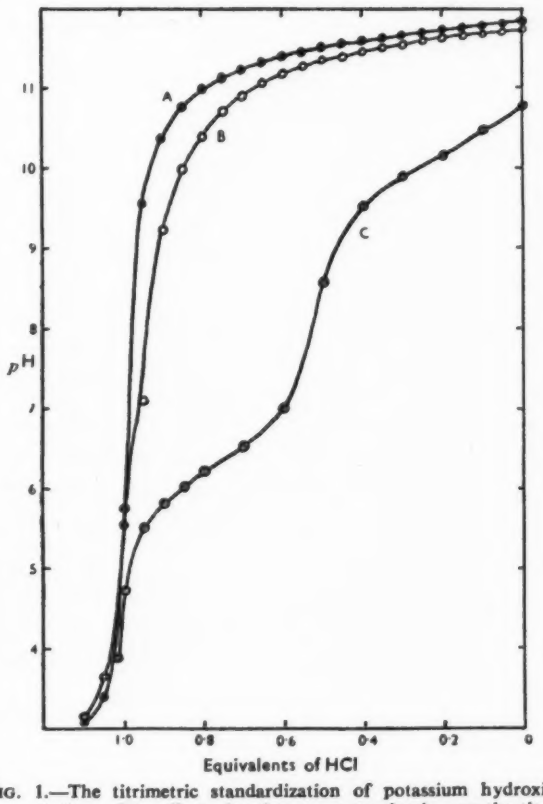


FIG. 1.—The titrimetric standardization of potassium hydroxide solution. The effect of carbonate contamination on the titration curve. A=pure KOH. B=KOH, 90%; Na_2CO_3, 10%. C=pure Na_2CO_3.

different groups of compounds (total range 0.20 to 0.545). The method described by Mizutani (1925) was therefore adopted, each compound being titrated in three different concentrations of alcohol (usually 30, 40, and 50%) and the values extrapolated back to zero % alcohol.

The dissociation constant (pK_a) was calculated from each pH reading on the titration curve according to the formula :

$$pK_a = pH - \log \frac{[B]}{[BH^+]}$$

the values for $[BH^+]$, the concentration of unchanged salt, and [B], the concentration of liberated free base, being derived from the titration figure. Where the pH readings were high, it was necessary to apply a correction for the concentration of free OH^- ions in solution, when the formula becomes :

$$pK_a = pH - \log \frac{[B] - [OH^-]}{[BH^+] + [OH^-]}$$

the values for $[OH^-]$ being derived from the observed pH. It is usual to apply this correction to all pH readings above 10, but, because of the low concentrations titrated, it was necessary to apply the correction to all readings above pH 9.

Determinations of pK_a values carried out on different samples of the same compound—in some cases at intervals of over two years—showed good agreement. For example, pK_a measurements on four samples of RP 3277 between 1950 and 1952 showed a range of 0.16 pK unit, and duplicate determinations on six other compounds differed by an average of 0.07 pK unit.

Results

Table II records the values for pK_a, pA_2, and pA_{10} for the compounds studied. Standard deviations of the values for pA_2 and pA_{10} are shown in parentheses. In a few of the "Wilson" series, technical difficulties prevented determinations of either pK_a or pA values. With compound 15, almost complete insolubility precluded either physical or pharmacological determinations, whereas with compound 13 the low pharmacological activity and relatively low solubility made it impossible to determine pA_{10}. With compounds A-2917 and A-4826 of the benzhydryl series (Table II) it was not possible to obtain characteristic titration curves even after conversion of the oxalate to hydrochloride. This might be expected from the structure of A-4826 (Table I), but it is hard to see why A-2917 should not show normal basic properties.

Distinction Between Competitive and Non-competitive Antagonists

Competitive and non-competitive antagonists may be distinguished by the difference between pA_2 and pA_{10}. When pA_{10} is measured, nine times as many molecules of histamine are being antagonized as when pA_2 is determined. If the antagonist functions by competing with histamine, it will require nine times as many molecules to antagonize a ninefold increase in histamine molecules, and the difference between pA_2 and pA_{10} will be ninefold, or 0.95 on the pA scale. If the antagonist is non-competitive, inhibiting only the contraction mechanism of the muscle, a smaller

TABLE II

pK_a AND pA_x VALUES

S.D. in parentheses. Type of antagonism: +, competitive; −, non-competitive; ⊕, probably competitive (see text)

Serial No. or Name	pK_a	pA_2	pA_{10}	(pA_2-pA_{10})	Type of Antagonism
"Wilson" Series					
1	8·37	3·91 (0·05)	3·25 (0·14)	0·66	−
2	9·04	4·06 (0·02)	3·56 (0·12)	0·50	−
3	8·30	4·70 (0·03)	4·22 (0·07)	0·48	−
4	9·40	4·91 (0·08)	4·43 (0·04)	0·48	−
5	8·80	4·65 (0·12)	3·88 (0·11)	0 77	+
6	9·03	5·13 (0·08)	4·56 (0·09)	0·57	−
7	6·23	3·33 (0·23)	2·75 (0·08)	0·58	−
8	6·17	4·24 (0·14)	3·78 (0·02)	0·46	−
9	8·71	5·46 (0·09)	4·65 (0·07)	0·81	+
10	9·55	5·25 (0·09)	4·64 (0·09)	0·61	−
11	9·45	5·39 (0·07)	4·74 (0·12)	0·65	−
12	6·39	4·50 (0·13)	3·96 (0·09)	0·54	−
13	10·25	3·19 (0·23)	Solubility exceeded		
14	9·38	5·15 (0·15)	4·55 (0·04)	0·60	−
15	Insoluble				
16	9·22	4·48 (0·22)	3·95 (0·09)	0·53	−
17	Ppt. insoluble in alcohol on titration				
18	9·07	5·33 (0·10)	4·09 (0·17)	1·24	⊕
19	8·99	5·26 (0·04)	4·34 (0·10)	0·92	+
20	8·90	5·04 (0·12)	4·57 (0·18)	0·47	−
20A	9·46	5·56 (0·08)	4·58 (0·11)	0·98	+
20B	Unstable				
21	8·99 7·13	5·09 (0·17)	4·45 (0·04)	0·64	−
22	9·68 7·18	4·86 (0·08)	4·32 (0·17)	0·54	−
23	9·99 7·98	5·53 (0·11)	4·52 (0·06)	1·01	+
24	10·09 8·41	4·63 (0·05)	3·82 (0·14)	0·81	+
25	9·78	4·44 (0 07)	4·04 (0 02)	0·40	−
Methadone Series					
50/XII/CN	9·08	5·73 (0·11)	4·73 (0·12)	1·00	+
49/III/CN(+)	8·68	5·44 (0·05)	4·84 (0·06)	0·60	−
" (−)	8·67	6·27 (0·23)	5·10 (0·10)	1·17	+
49/IV/CN	8·16	5·14 (0·08)	4·50 (0·10)	0·64	−
50/II/CN	8·93	6·12 (0·08)	4·86 (0·11)	1·26	⊕
50/I/CN	8·83	5·31 (0·11)	4·24 (0·15)	1·07	+
50/XII/COOH	10·59	3·14 (0·22)	2·43 (0·04)	0·71	+
49/III/COOH	10·88	3·64 (0·05)	2·67 (0·03)	0·97	+
49/III/COOEt	10·12	5 41 (0·18)	5·02 (0·19)	0·39	−
50/XII/COOEt	11·59	5·00 (0·16)	4·04 (0·08)	0·96	+
49/V/COOEt	9·87	5·52 (0·05)	4·93 (0·16)	0·59	−
49/III/COBu	10·38	5·65 (0·19)	5·24 (0·21)	0·41	−
49/IV/COMe	9·53	5·01 (0·13)	4·76 (0·14)	0·25	−
50/II/COEt	10·35	5·68 (0·06)	5·09 (0·08)	0·59	−
50/I/COEt	9·40	5·65 (0·11)	4·75 (0·22)	0·90	+
W17	8·65	3·25 (0·13)	2·51 (0·01)	0·74	−
W18	9·67	6·04 (0·20)	5·15 (0·18)	0·89	+
Methadone	10·12	5·78 (0·20)	4·84 (0·18)	0·94	+
17	6·96	4·60 (0·03)	4·28 (0·15)	0·32	−
Phenadoxone	7·70	5·07 (0·20)	4·62 (0·22)	0·45	−
*iso*Phenadoxone	7·12	4·72 (0·29)	4·27 (0·10)	0·45	−
16	6·83	4·52 (0·10)	4·00 (0·11)	0·52	−
2	8·96	5·59 (0·05)	4·97 (0·14)	0·62	−
18	7·17	4·67 (0·11)	4·51 (0·09)	0·16	−
4	9·47	5·79 (0·07)	5·24 (0·09)	0·55	−

TABLE II—*continued*

Serial No. or Name	pK_a	pA_2	pA_{10}	(pA_2-pA_{10})	Type of Antagonism
Proprietary Antihistamines					
Benadryl (diphenhydramine)	8·98	8·14 (0·13)	6·78 (0·19)	1·36	⊕
Pyribenzamine (tripelennamine)	8·95	9·00 (0·24)	7·64 (0·13)	1·36	⊕
Anthisan (mepyramine)	8·85	9·36 (0·26)	8·38 (0·12)	0·98	+
Antistin	10·00	7·67 (0·13)	6·39 (0·19)	1·28	+
Histostab	9·97	7·40 (0·20)			
Chlorothen	8·70	9·50 (0·08)	8·35 (0·12)	1·15	⊕
Bromothen	8·63	9·64 (0·27)	8·26 (0·04)	1·38	⊕
Phenergan (promethazine)	9·08	8·93 (0·10)	7·96 (0·06)	0·97	+
Histantin		8·80 (0·18)			
Di-paralene (chlorcyclizine)	8·15	8·63 (0·08)	7·14 (0·25)	1·49	⊕
Trimeton (prophenpyridamine)	9·23	7·82 (0·24)	6·74 (0·05)	1·08	+
Chlor-trimeton (chlorprophenpyridamine)	9·16	8·82 (0·13)	7·89 (0·17)	0·93	+
Thenylene (methapyrilene)	8·85	8·63 (0·11)	7·64 (0·09)	0·99	+
Thephorin (phenindamine)	8·98	8·46 (0·21)	7·20 (0·18)	1·26	⊕
Phenothiazine Derivatives					
3015 RP	8·66	8·76 (0·17)	8·15 (0·16)	0·61	−
2987 RP	9·09	7·88 (0·05)	7·17 (0·08)	0·71	−
Halpern	9·02	7·95 (0·16)	7·17 (0·03)	0·78	+
3277 Phenergan	9·08	8·93 (0·10)	7·96 (0·06)	0·97	+
RP	9·06	8·86 (0·13)	8·26 (0·23)	0·60	−
Halpern	8·92	8·78 (0·23)	7·96 (0·10)	0·82	+
Lergigan	8·97	8·89 (0·16)	7·61 (0·16)	1·28	⊕
4460 RP	8·91	7·97 (0·07)	7·13 (0·27)	0·84	+
3356 RP	9·50	8·34 (0·11)	7·31 (0·04)	1·03	+
Halpern	9·64	8·27 (0·09)	7·31 (0·03)	0·96	+
3349 RP	9·61	6·50 (0·23)	5·79 (0·12)	0·71	+
4605 RP	9·02	7·60 (0·11)	7·06 (0·12)	0·54	−
4588 RP	8·96	7·81 (0·20)	7·16 (0·18)	0·65	+
3554 RP	11·23	8·18 (0·20)	7·81 (0·09)	0·37	−
3276 RP	9·52	8·25 (0·14)	7·39 (0·25)	0·86	+
3300 RP	9·13	7·00 (0·20)	6·26 (0·15)	0·74	+
Halpern	9·08	7·14 (0·07)	6·27 (0·07)	0·87	+
4560 RP	9·30	7·92 (0·11)	6·93 (0·20)	0·99	+
Benzhydryl (Chlorcyclizine) Series					
A-1198 ..	8·16	7·87 (0·07)	6·80 (0·06)	1·07	+
A-2285 ..	7·97	8·15 (0·09)	7·17 (0·05)	0·98	+
A-2342 ..	8·23	8·03 (0·09)	7·14 (0·04)	0·89	+
A-2688 ..	8·21	8·16 (0·11)	7·16 (0·23)	1·00	+
A-2824 ..	8·10	7·25 (0·22)	6·22 (0·10)	1·03	+
Chlorcyclizine	8·15	8·63 (0·08)	7·14 (0·25)	1·49	⊕
A-2917	Not titratable	7·09 (0·17)	5·93 (0·05)	1·16	⊕
A-4826	Not titratable	5·23 (0·15)	4·07 (0·14)	1·16	+
Phenindamine and Derivatives					
Phenindamine	8·98	8·46 (0·21)	7·20 (0·18)	1·26	⊕
Nu-1326 ..	7·71	6·39 (0·11)	5·26 (0·07)	1·13	⊕
Nu-1525 ..	8·66	5·71 (0·15)	4·61 (0·20)	1·10	+

increase in antagonist will be required to antagonize a ninefold increase in spasmogen concentration, since the increase in *contraction* is always much less than ninefold. This relationship between type of antagonism and (pA_2-pA_{10}) difference has been described by Schild (1947), and can be derived mathematically from the Mass Action equation of Gaddum (1937).

The frequency distribution of (pA_2-pA_{10}) differences for all the compounds tested (Fig. 2) shows two clearly defined peaks at 0.60 and 0.95. The latter peak most likely represents the competitive compounds, whereas the peak at 0.60 represents the non-competitive compounds. A method of dividing the compounds statistically into the two groups was devised by adding 0.95 to each pA_{10} value and then determining whether or not this sum differs significantly from the value for pA_2 by calculating the value of t (P=0.05). The criterion of competitive or non-competitive action denoted in the final column of Table II is based on this calculation.

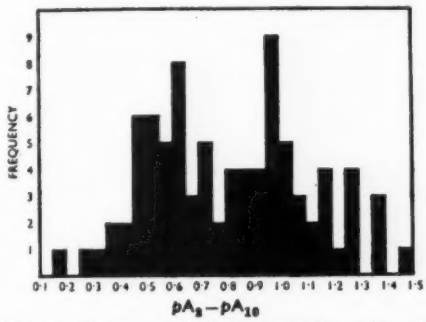


FIG. 2.—Frequency distribution of the values of pA_2-pA_{10}, showing peaks at 0.60 and 0.95.

Abnormally High (pA_2-pA_{10}) Differences

Fig. 2 shows that there is considerable spread of values for (pA_2-pA_{10}) above 0.95, the theoretical value for competitive antagonism. Some of these high values fall outside the calculated limits of deviation from 0.95, and such compounds are therefore theoretically non-competitive. On logical grounds, however, there is no justification for accepting such compounds as non-competitive, particularly as a large proportion of the proprietary antihistamines are in this category. As many of the antihistamines also antagonize ACh, a possible explanation of the abnormally high (pA_2-pA_{10}) values suggested itself, and the theory was to some extent substantiated by experimental evidence.

It was noted that mepyramine had a normal (pA_2-pA_{10}) difference, whereas that of diphenhydramine was high. It is well known that, although diphenhydramine shows powerful anti-ACh as well as antihistamine action, mepyramine is a relatively specific antihistamine. If, therefore, diphenhydramine molecules have an affinity for both histamine and ACh receptors, it might be that, at the relatively higher concentrations of diphenhydramine used in determining pA_{10} against

histamine, a higher proportion of diphenhydramine molecules are attracted to ACh receptors. There would therefore be increased competition between the two types of receptor for diphenhydramine molecules, so that the apparent amount of drug required to produce a given antagonism of histamine would be greater than if the drug were taken up solely by histamine receptors. Thus the pA_{10} value would be lower, and the (pA_2-pA_{10}) difference greater. Furthermore, if this theory is true, then it should be possible to restore the (pA_2-pA_{10}) difference to the theoretical 0.95 by determining pA_2 and pA_{10} values in atropinized Tyrode solution, since then the ACh receptors would already be blocked by the atropine, leaving all the diphenhydramine molecules free to combine with histamine receptors.

Accordingly, the pA_2 and pA_{10} values for mepyramine—normal (pA_2-pA_{10}) difference—and three antihistamines having high (pA_2-pA_{10}) differences were re-determined, using Tyrode solution containing 10^{-6} atropine sulphate, and the new (pA_2-pA_{10}) differences were compared with those previously obtained with normal Tyrode solution (Table III). With mepyramine the

TABLE III

EFFECT OF ATROPINIZED TYRODE ON THE (pA_2-pA_{10}) DIFFERENCE. (EACH pA VALUE QUOTED IS THE MEAN OF FOUR DETERMINATIONS)

Antihistamine	Normal Tyrode			Tyrode + Atropine SO_4 10^{-6}		
	pA_2	pA_{10}	(pA_2-pA_{10})	pA_2	pA_{10}	(pA_2-pA_{10})
Mepyramine ..	9·36	8·38	0·98	8·95	7·93	1·02
Diphenhydramine	8·14	6·78	1·36	7·68	6·52	1·16
Tripelennamine	9·00	7·64	1·36	8·49	7·33	1·16
Chlorcyclizine ..	8·63	7·14	1·49	7·98	6·90	1·08

value was unchanged, but with diphenhydramine, tripelennamine, and chlorcyclizine the original high values were reduced, though not to the theoretical 0.95. These results give some support to the theory of the origin of high (pA_2-pA_{10}) values. The slopes of the pA_2 and pA_{10} regression lines, determined in normal and atropinized Tyrode, differed significantly only for chlorcyclizine; that is, only with chlorcyclizine was the reduction of (pA_2-pA_{10}) difference significant. With chlorcyclizine and tripelennamine, but not diphenhydramine, the (pA_2-pA_{10}) difference, when determined in atropinized Tyrode, did not differ significantly from 0.95.

It is, therefore, considered justifiable to designate all compounds with abnormally high (pA_2-pA_{10}) difference as competitive antagonists of histamine. They are distinguished in Table II (last column) from the statistically proven competitive antagonists by the sign ⊕.

Duplicate Samples

Where more than one sample or proprietary preparation of the same compound was included, the pK_a values were always in good agreement, and there was no significant difference between the pA_2 values of eight compounds in which duplicate samples were tested. With regard to the type of histamine antagonism, discrepancies occurred between samples of RP 3277 and 2987. Whereas promethazine, lergigan and Halpern 3277 appeared to be competitive antagonists of histamine, the Rhone-Poulenc sample of 3277 was non-competitive. It was thought that this discrepancy might be explained by the latter sample containing a larger proportion of the (−)-isomer, since in compound 49/III/CN (Table II) the (−)-isomer is competitive and the (+)-isomer non-competitive. However, when examined polarimetrically, promethazine, RP 3277 and Halpern 3277 were all found to be optically inactive (Burke, private communication). Melting point determinations (Wragg, private communication) also failed to suggest any reason for the discrepancy. In comparison with a recent batch of promethazine (M. and B., batch 76, m.p. 233° C.), RP 3277 and Halpern 3277 were of reasonable purity (m.p. 232° C. and 231° C., respectively), while lergigan is slightly impure (m.p. 228° C.) and the original sample of promethazine very impure (m.p. 214–226° C.). It is strange, therefore, that the relatively pure RP 3277 is apparently non-competitive, while the less pure promethazine and lergigan are competitive.

Relation Between pA_2 and pK_a

Early studies on the proprietary antihistamines indicated a correlation between activity and dissociation constant. The most active antihistamine tested was bromothen which has a pK_a value of 8.63. Compounds with pK_a values below or above this were less potent, and above 8.63 the potency decreased inversely with the pK_a value. Unfortunately only one compound, chlorcyclizine, fell below pK_a 8.63, but this had a proportionately lower antihistamine potency than bromothen. From these data it was possible to construct, as a working hypothesis, a curve relating antihistamine potency to pK_a and resembling that obtained by Bell and Roblin (1942) for the sulphonamides (Fig. 3).

Determinations of antihistamine potencies more accurately in terms of pA units gave essentially the same picture for the proprietary antihistamines

as that obtained in the preliminary experiments. The investigation was then extended to a wide range of compounds all of which inhibited histamine-induced contractions of plain muscle. It was soon realized that these compounds must be screened not only for antihistamine potency but also to determine which were competitive antagonists of histamine, since it would be incorrect to correlate antihistamine potency with pK_a in compounds which were not competitive antihistamines. The results of this investigation have already been recorded in Table II, in which compounds with a (pA_2-pA_{10}) difference not significantly different from 0.95 are regarded as being competitive. Correlation coefficients between pA_2 and pK_a were then determined for the competitive and non-competitive members of each chemical group. These are summarized in Table IV. Assuming that a peak activity occurs at pK_a 8.6, coefficients have been calculated separately for compounds below 8.6 (positive correlation) and for compounds above 8.6 (negative correlation). As in the early experiments, the majority of compounds among the competitive antagonists had a pK_a value higher than 8.6. With the exception of Nu-1326, only the compounds related to chlorcyclizine had values below 8.6. The results show that there is a significant correlation between pA_2 and pK_a for the proprietary antihistamines with pK_a values above 8.6

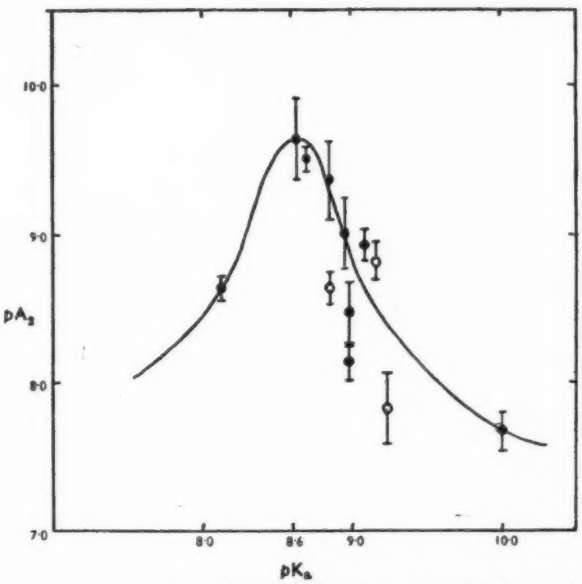


FIG. 3.—Curve of the relationship between pK_a and pA_2 for the proprietary antihistamines. (Vertical lines represent standard deviations of pA_2 values.) Solid dots—nine antihistamines upon which the curve was drawn. Open dots—three antihistamines (trimeton, chlor-trimeton, and thenylene) added after the hypothetical curve had been constructed.

TABLE IV
RELATIONSHIP BETWEEN pA_2 AND pK_a

Series	Correlation			
	No. of Compounds	Coefficient r	Tabulated r (P=0·05)	Significance
	Competitive compounds			
Proprietary antihistamines (above pK_a 8·6) ..	11	−0·78	0·59	+
Benzhydryls (below 8·6)	6	+0·11	0·76	−
"Wilson" (above 8·6)..	7	−0·069	0·72	−
Phenothiazines (above 8·6)	13	−0·51	0·55	−
"Methadone" (above 8·6)	10	−0·70	0·63	+
All competitive comps. (above 8·6)	41	−0·50	0·30	+
All competitive comps. (below 8·6)	7	+0·76	0·72	+
	Non-competitive compounds			
"Wilson" (above 8·6)..	11	+0·018	0·59	−
"Wilson" (below 8·6)..	5	+0·30	0·84	−
Phenothiazines (above 8·6)	5	−0·16	0·84	−
"Methadone" (above 8·6)	9	+0·57	0·65	−
"Methadone" (below 8·6)	6	+1·15	0·76	+
All non-competitive comps. (above 8·6) ..	25	+0·11	0·40	−
All non-competitive comps. (below 8·6) ..	11	+0·43	0·59	−

(all of which were competitive), for the competitive compounds in the "methadone" series with pK_a values above 8.6, and for the total competitive compounds with pK_a values above and below 8.6. Among the non-competitive compounds, only one group, the "methadone" series below 8.6, showed significant correlation. The significance of correlation was never altered by inclusion or exclusion of the compounds with abnormally high (pA_2-pA_{10}) difference.

DISCUSSION

The evaluation of antihistamine potency in a wide range of compounds has emphasized the value of the *p*A method of assay, both as regards quantitative accuracy and the qualitative division of the compounds into groups representing competitive and non-competitive antagonists. When the frequency distribution of compounds according to the difference between pA_2 and pA_{10} values is plotted, two clearly marked peaks are obtained, one at *p*A 0.60, and the other at *p*A 0.95. The lower value represents fourfold, and the higher value ninefold, increase in the amount of antagonist required to inhibit a ninefold histamine increase. This latter reflects the molecule-for-molecule relationship which characterizes competitive antagonism, expressed mathematically in the Mass Action equation of Gaddum (1937). Any (pA_2-pA_{10}) value significantly lower than 0.95 must represent an antagonism which is non-competitive, and due to general spasmolytic action

on the plain muscle contraction rather than to a competition for histamine receptors. Some compounds have shown (pA_2–pA_{10}) differences significantly higher than 0.95, and it is suggested that these high values are related to those compounds which are competitive antagonists of ACh as well as of histamine, so that, at higher concentrations, some of the antagonist is "wasted" by being adsorbed on to ACh receptors. In support of this theory it has been shown that, in three of the compounds with high values, the pA_2–pA_{10} difference can be lowered by blocking ACh receptors with atropine. This multiple affinity for different types of receptor might explain the high values obtained by Guarino and Bovet (1949), who derived an equation which required a 45-fold increase of antagonist to balance a ninefold increase of active drug. This represents a (pA_2–pA_{10}) difference of 1.65. The highest value obtained for the antihistamines reported in this paper was 1.49 (that is, 30-fold) for chlorcyclizine.

The main purpose in dividing the compounds into competitive and non-competitive groups was to test the validity of a relationship between antihistamine potency and dissociation constant which had been previously observed in the proprietary antihistamines. The most potent antihistamine tested has a pK_a of 8.63, those above and below this value having lower activity, so that it was assumed as a working hypothesis that the relation between antihistamine potency and pK_a followed a curve, with a maximum activity at pK_a 8.6. This hypothesis has been tested on the results obtained from a wide range of spasmolytic compounds inhibiting histamine, by calculating correlation coefficients for pA_2 against pK_a for competitive and non-competitive compounds above and below pK_a 8.6. Taking all the compounds in each of these four groups, it has been shown that there is a significant negative correlation below pK_a 8.6 and a significant negative correlation above pK_a 8.6 for the competitive compounds. On the other hand, there is no correlation between pA_2 and pK_a in the non-competitive groups. When the compounds are divided into smaller, chemically related groups, the relationship does not always hold good, possibly because of insufficient numbers in some groups. These results strongly support the suggestion that the pA_2–pK_a relationship for antihistamine compounds follows a curve similar to that of Bell and Roblin (1942) for the sulphonamides.

The validity of this relationship has been tested by predicting the pA_2 values of three antihistamines, the dissociation constants of which were determined before their antihistamine potency. From the pA_2–pK_a curve plotted from data obtained with nine other proprietary antihistamines, the theoretical pA_2 value was read off according to the pK_a value for each compound. The predicted and experimentally determined values for pA_2 for the compounds were: prophenpyridamine, 8.12; 7.82 (0.24): chlorprophenpyridamine, 8.22; 8.82 (0.13): methapyrilene, 9.20; 8.63 (0.11). Figures in parentheses are standard deviations. This shows reasonably good agreement between predicted and actual values.

The division of a relatively large number of compounds into competitive and non-competitive antagonists of histamine has provided some interesting observations on the chemical structures required for histamine competition. Usually a two-carbon side-chain is important for specific antihistamine action, but duplicating this ethylamine side-chain as in compound 20 destroys the competitive action. Addition of other nitrogenous basic groups to the molecule (compounds 21, 22) also nullifies the action of the ethylamine chain, but in compounds 23 and 24, where the additional nitrogenous side-chain forms a closed ring, competitive antagonism is maintained.

Modification of the ethylamine side-chain is possible, however, within certain limits without destroying competitive action. The "methadone" series provides an illustration of the extent to which such modification can be made. Substituting a piperidine ring for the terminal amine does not affect the competition with histamine (compounds 50/II/CN, 50/I/CN, 50/I/COEt), but substituting a morpholine ring as in phenadoxone and related compounds destroys competitive action. The reduction of antihistamine and spasmolytic potency on substituting the morpholine group has previously been reported (Forbes and Marshall, 1951). Addition of a methyl group to one or other of the two carbon atoms of the side-chain has variable effects, apparently depending on the structure of the rest of the molecule. Thus, in compounds 49/IV/CN and 49/IV/COMe, substitution on the β-carbon atom renders the compound non-competitive, whereas in compounds 50/I/CN and 50/I/COEt histamine competition is maintained in the presence of the β-methyl substitution. It may be significant that compounds 50/I/CN and 50/I/COEt both have the piperidine nitrogen. In compounds 49/III/CN, 50/II/CN, 49/III/COOH and methadone itself, competitive action is maintained in the presence of α-methyl substitution, whereas compounds 49/III/COOEt, 49/III/COBu, and 50/II/COEt, also with α-methyl groups, are non-competitive. In the phenothiazine series, both the

α- and β-methyl derivatives (as in promethazine, 3277 and *iso*-promethazine, 4460) are competitive. Substitution by ethyl in the α-position (compound 4605) destroys histamine competition.

Obviously, all compounds with an ethylamine side-chain are not antihistamines, and the structure of the nucleus of the molecule plays an important part in establishing competitive antagonism. The "methadone" series provides an interesting illustration of this point, though it is impossible to establish any regular rule with regard to the function of the various substituents on the second (non-basic) side-chain in rendering the compound competitive or not. It appears that specificity for histamine is decided by a "balance" between the substituents on the ethylamine side-chain and on the rest of the molecule. For example, compounds 49/V/COEt, 49/III/COBu, 49/IV/COMe, and 50/II/COEt with a ketonic second side-chain are non-competitive, but the same ketonic side-chain associated with β-methyl and pyrimidine nitrogen substitution in the ethylamine chain (compound 50/I/COEt) or α-methyl substitution (methadone) renders the compound competitive.

The two stereo-isomers of 49/III/CN are interesting, the (+)-isomer being non-competitive and the (−)-isomer competitive. Unfortunately no other resolved compounds were available, so that it was impossible to establish whether (−)-rotation is always essential for histamine specificity in optically active compounds. An anomaly is seen with quaternized compounds. Compound 50/XII/COOEt ("methadone" series) was supplied as the methyl iodide, and was competitive; on the other hand, quaternization of promethazine (compound 3554) results in loss of competition for histamine.

Several apparent anomalies occur in the phenothiazine series. Of four samples of RP 3277 from three different sources, one is non-competitive and the other three competitive. The sample of lergigan, supplied originally under the formula of compound 4460, but since identified as identical with promethazine, was competitive. A similar difference in type of inhibition occurs between the two samples of compound 2987. These anomalies are not explicable on grounds such as difference in optical rotation or in purity.

Another unusual feature of the phenothiazine series is that compound 4560, with a three-carbon side-chain, is competitive. Here, the chlorine atom on the phenothiazine ring may be a factor "balancing" the extra carbon atom in the side-chain.

One group of compounds, the phenindamine series, deviates considerably from the typical antihistamine structure. Phenindamine itself, with a pK_a of 8.98, falls reasonably close to the pA_2–pK_a curve. It is a competitive antagonist, as also are the two related compounds Nu-1326 and Nu-1525. One would therefore expect Nu-1525 (pK_a 8.66) to be very potent, and Nu-1326 (pK_a 7.71) to have low antihistaminic potency, but the pA_2 of Nu-1525 is only 5.71, whereas that of Nu-1326 is 6.39. Although only three compounds in this series were available, one is tempted to suggest that the mechanism of antihistamine action of these compounds is different from that of other antihistamines, and is probably not a function of ionization.

The conclusions to be drawn from this study are that two factors are involved in determining antihistamine activity. First of all, qualitatively, the structure and shape of the molecule determines whether or not the compound is a competitive antagonist of histamine. The relative structure of both ring and side-chain appear to be important, and it is only possible, from observations on a relatively small number of compounds, to make tentative suggestions as to the limitations of structure capable of conferring antihistamine activity. The second factor is the basicity of the nitrogenous group of the molecule, which appears to exert a quantitative control on the potency of competitive inhibitors of histamine. Thus, if a compound possesses the right structure for antihistamine action, then the nearer its dissociation constant is to pK_a 8.6, the more potent it will be.

Summary

1. A wide range of nitrogenous basic compounds has been screened for antihistamine potency by the pA method. Both pA_2 and pA_{10} were determined in order to distinguish competitive antagonists of histamine from non-competitive spasmolytics.

2. The dissociation constants (pK_a) of the compounds were measured by potentiometric titration, and a significant relationship between dissociation constant and antihistamine potency was established for those compounds which are competitive.

3. Factors concerning the relationship between chemical structure and antihistamine action are discussed in the light of the results obtained. It is suggested that a different mode of action operates with derivatives of phenindamine.

The author is indebted to the following individuals and organizations for supplying compounds:

Dr. G. W. Anderson, American Cyanamid Co. (chlorothen and bromothen); Burroughs Wellcome and Co. (histantin, methadone); Abbott Laboratories, Ltd. (di-paralene and chlorcyclizine derivatives; thenylene); Boots Pure Drug Co. (histostab); May and Baker, Ltd. (mepyramine; promethazine); Ciba

Laboratories, Ltd. (tripelennamine ; antistin) ; Dr. W. Wenner, Hoffman-La Roche (thephorin and phenindamine derivatives) ; Dr. W. Wilson, Chemistry Dept., University of Birmingham ; Dr. H. M. Walker, Glaxo Laboratories, Ltd. (phenadoxone and derivatives) ; Dr. E. Walton, Wellcome Chemical Laboratories (" methadone " series) ; Dr. B. N. Halpern (phenothiazines ; prophenpyridamine and chlorprophenpyridamine) ; Rhone-Poulenc, Paris (phenothiazines).

The author also wishes to thank Professor Adrien Albert for advice on the determination of dissociation constants, Dr. Burke (University of Birmingham) for measurements of optical rotation in phenothiazines, Dr. W. R. Wragg (May and Baker, Ltd.) for determinations of melting points of samples of promethazine, and Professor A. C. Frazer for his interest in the progress of the work.

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THE DEVELOPMENT OF TOLERANCE AND CROSS-TOLERANCE TO METHONIUM COMPOUNDS IN LABORATORY ANIMALS

BY

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(RECEIVED JULY 27, 1954)

Since the discovery of the ganglion-blocking action of hexamethonium and pentamethonium salts by Paton and Zaimis (1949), and a study of their clinical pharmacology by Arnold and Rosenheim (1949), these drugs have been used extensively in clinical practice, particularly in the treatment of hypertension (Restall and Smirk, 1950; Turner, 1950; Saville, 1950; Campbell and Robertson, 1950; Smirk and Alstad, 1951; Freis, 1951; McMichael, 1952; Morrison, 1953).

Smirk (1950) and Smirk and Alstad (1951) drew attention to the development of tolerance to pentamethonium and hexamethonium with their continued administration. In later communications Smirk (1952, 1953) reported cross-tolerance between these substances and between certain of their chemical relatives. As the development of such tolerance is a distinct disadvantage clinically—in that it is not possible to arrive at a stable dose level for several months, and hence necessitates continuous and close observation of the patient—the phenomenon has been studied experimentally. Some of the results have been mentioned in a preliminary communication (Mohanty, 1954).

METHODS

Toxicity Studies.—White mice aged 6–8 weeks and of homogeneous stock were used. The animals were divided into two groups by random sampling. One group received daily subcutaneous injections of hexamethonium bromide (C6) for a period of 10 days, the daily dose being increased gradually over this period. The other group received daily subcutaneous injections of 0.9% NaCl in equal volume. Next, 48 hr. after the last injection for the 10-day period, the mice of each group were subdivided by random sampling into 4 dose groups. Graded doses of C6 were given to mice of each of the 8 groups, suitable doses having been found previously by pilot experiments. The dose was proportional to the body weight and was given subcutaneously. Death or survival was recorded at the end of 6 hr.

Measurement of Mydriatic Effects.—The method used was essentially the same as that described by Ing, Dawes, and Wajda (1945). White mice aged 6–8 weeks and of homogeneous strain were kept under standard illumination for 30 min. before the measurement of the initial pupillary size and the injection of the drug. Pupillary diameters were again measured, 10 to 20 min. after the injection, by a microscope with an arbitrary scale in the eye-piece. In the first set of experiments, the dose-response curves were determined for the following compounds: C6, M&B 2050 [pentamethylene 1:5-bis-*N*-(*N*-methyl-pyrrolidinium) di-iodide], M&B 1863 [hexamethylene 1:6-bis-(ethyldimethylammonium) bromide], M&B 1950 [*N*, *N'*-tetramethyl-β-*p*-aminophenylethylamine) di-iodide], TEAB (tetraethylammonium bromide), SC-2159 and SC-1718. The last two compounds were kindly supplied by G. D. Searle & Co., and the remaining compounds by Messrs. May & Baker.

Fresh mice were assigned by random sampling into groups of 15 animals, one such group being used to test each compound. A test dose lying in the middle portion of the log dose-response curve for that substance was selected. Daily subcutaneous injections were given for 6–8 days. Changes in pupillary size were measured after each injection. A "cross-over" test was performed at the end of this period. Cross-tolerance was investigated mostly with C6 as reference standard drug.

Experiments with Isolated Intestine.—Trendelenburg's (1917) preparation was employed. Peristalsis was induced by raising the intraluminal pressure by 2.5 cm. of water. Intestinal strips from normal guinea-pigs and rabbits were used to determine the doses of the various methonium compounds which would produce paralysis of the peristaltic movement. To study tolerance and cross-tolerance, similar preparations were made with intestinal strips from guinea-pigs and rabbits which had been made tolerant by gradually increased daily subcutaneous injections of C6 or

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TEAB. Animals were normally sacrificed 48–72 hr. after the last injection for the 14-day period; but preparations from 3 tolerant guinea-pigs were examined at 1 week, 2 weeks, and 2 months (respectively) after the final injection in order to discover whether tolerance persisted. The preparations were taken from the lower part of the ileum and suspended in Ringer-Tyrode solution at 35° C. In all, 77 experiments were performed with this preparation.

Experiments with the Cat's Superior Cervical Ganglion.—Animals were anaesthetized with intraperitoneal injections of pentobarbitone-urethane. The nictitating membrane was prepared for recording in the usual way (Burn, 1952). Usually the experiment was elaborated by performing such operations as the following:

(1) It was often desirable to mount both nictitating membranes for simultaneous recording of their contractions, in order to demonstrate differences between the effects of C6 on a treated ganglion and on a control.

(2) Many experiments were performed with a vascularly isolated perfused ganglion preparation. In such experiments the superior cervical ganglion was dissected out and isolated from the rest of the body but for its nervous and vascular connexions; then the homolateral carotid artery was cannulated and perfused at a constant rate with a blood substitute. A rotary pump—the F.31 model of Messrs. C. F. Palmer (London), Ltd.—driven at slow speed was used for this purpose. Perfusion pressure and the contractions of the nictitating membrane were recorded. The perfusion medium was that described by Gallagher (1954). It was made by suspending washed sheep or human red cells in Krebs-Henseleit solution to which had been added 6% dextran and 0.1% glucose. The *p*H of the solution was kept at 7.2–7.4. The dose of C6 injected into the perfusion cannula was calculated to give a concentration in the perfusion fluid as great as, or greater than, that which would be given by the intravenous test dose. The injection was given at the height of contraction of the nictitating membrane. In most experiments the intravenous dose of C6 was 5 mg./kg. of the bromide and the intra-arterial dose 0.1–0.5 mg. Blood pressure was recorded from a carotid artery. Intravenous injections were made into a femoral vein.

(3) In some experiments, the ganglion was perfused for a time with heparinized blood obtained from untreated cats or patients in place of the simple blood substitute. In others, the perfusion medium was changed for 15–60 min. from the simple blood substitute to blood or plasma taken from cats or patients treated previously with C6.

The patients were some of the hypertensives under treatment with C6 at Dunedin Public Hospital. Details of their treatment have been published elsewhere (Smirk, 1953). All of those from whom blood was taken had become tolerant to C6 and were receiving at least 50 mg. of the bromide daily by subcutaneous injection, but no C6 was administered during the 24 hr. period before the removal of the blood. Immediately after collection the blood was heparinized. It was then centrifuged and the plasma collected. This plasma was mixed with the plain blood substitute in the proportion of 1:3.

The "C6-tolerant cat's blood" was obtained from cats that had been given daily subcutaneous injections of C6 in gradually increasing doses (5–20 mg./kg.) for a period of 14 days. Two to three days after the last injection they were anaesthetized, their carotid arteries were cannulated with polythene tubing, and the blood from them was collected in a paraffined beaker. Heparin was added to it.

Blood from normal cats, and blood and plasma from normal patients, were obtained in a similar fashion for control experiments.

(4) The procedures for applying C6 locally to a superior cervical ganglion were: (*a*) Both ganglia were exposed and isolated but for their nervous and vascular connexions. One ganglion was then surrounded by pledgets of cotton-wool soaked in 0.9% NaCl to which C6 (1%) had been added. The other ganglion was treated similarly, but with 0.9% NaCl alone. During the next 24 hr. C6 was applied continuously to the one ganglion and the plain physiological saline to the other. Lastly, both ganglia were washed with Ringer-Locke solution for some 30–60 min. before the pre-ganglionic nerves were again stimulated and the effects of an intravenous injection of C6 given at the height of contraction of the nictitating membrane recorded. (*b*) Alternatively, one ganglion was perfused for 1 hr. with blood substitute containing 5 μg./ml. of C6 and for a further 30–60 min. with the simple blood substitute before the test doses of C6 were repeated.

Results

Acute Toxicity of Hexamethonium for Normal and for C6-treated Mice

In both the C6-treated and untreated mice the test injection of C6 was followed within a few minutes by asthenia and flaccidity, due apparently to loss of muscular tone. Respiration became shallow and rapid, and the pupils widely dilated. Death occurred as the result of respiratory paralysis and was preceded in some mice by a short period of convulsions, probably due to asphyxia.

The results given in Table I were analysed graphically by the method of Litchfield and Fertig (1941). The LD50 for the untreated mice was found to be 148.0 mg./kg. with 95% fiducial limits of 144.3 and 151.6 mg./kg.; the LD50 for the C6-treated mice was 160.0 mg./kg. with 95% fiducial limits of 147.2 and 173.9 mg./kg. The difference between these LD50's is therefore not significant at the 95% level. The observed difference in slope of the regression lines was also not significant—for the untreated mice "b" was 23.6 with S.E. of 8.81 and for the treated mice it was 8.20 with S.E. of 5.65 (significance of difference,

TABLE I

ACUTE TOXICITY OF HEXAMETHONIUM IN NORMAL AND IN HEXAMETHONIUM-TREATED MICE

	Normal Mice				Tolerant Mice			
Dose of C6 (mg./kg.) ..	140	145	150	155	140	150	160	170
Log. dose	2·146	2·162	2·176	2·190	2·146	2·176	2·204	2·230
Mortality	9/30	12/30	17/30	21/30	7/20	8/20	10/20	12/20
% mortality ..	30	40	56·6	70	35	40	50	60
Probits	4·48	4·75	5·17	5·52	4·61	4·75	5·00	5·25

P=0.14). It therefore seems that little tolerance develops to the lethal action (or actions) of hexamethonium.

Tolerance and Cross-tolerance to the Mydriatic Action of Methonium Compounds in Mice

The dose-response curves obtained with various onium compounds are illustrated in Fig. 1. They are non-linear, with the exception of the dose-response curve for SC-1718. The linearity of the dose-response curve was not improved by plotting the dose against the increase in pupil diameter instead of against the final diameter (Fig. 1). Four separate determinations of the dose-response curve for C6 indicated that results were closely reproducible. The dose-response curves for all the other drugs except TEAB are almost parallel to that for C6 with the dose ranges tried.

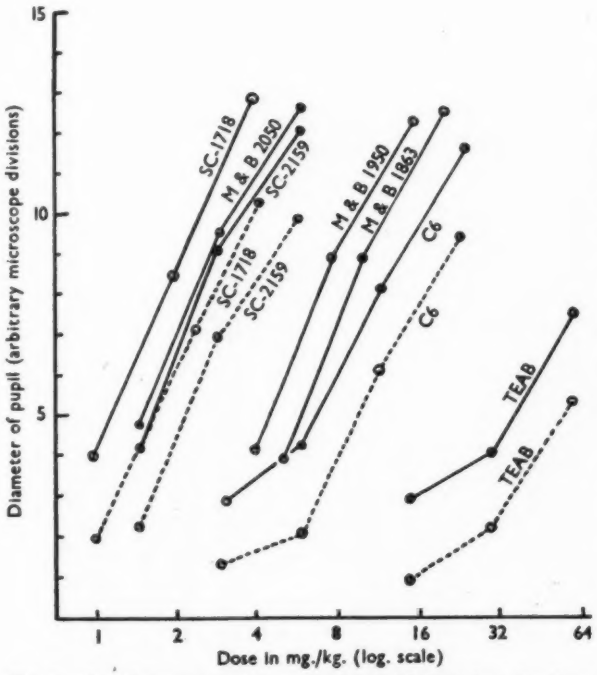

FIG. 1.—Log dose-response curves for the mydriatic action of various methonium compounds in mice. A broken line indicates that allowance has been made for the initial size of the pupil. See "Methods" for structures of drugs.

Tolerance was observed with all the compounds except SC-1718 (Figs. 2 and 3). The greatest degree was observed with C6. With the remaining drugs the degree of tolerance under comparable conditions decreased in the order: M&B 1950, SC-2159, M&B 1863, M&B 2050, TEAB (Fig. 3). Some tolerance could be detected after the first injection and, with continued daily injections, tolerance developed progressively until the completion of the experiment. Cross-tolerance between C6 and M&B 1950, M&B 2050, M&B 1863, and SC-2159 was observed. M&B 1863 gave rise to most tolerance to C6, followed (in descending order of potency) by SC-2159, M&B 2050, and M&B 1950. Mice first treated with C6 showed most tolerance to TEAB, followed by M&B 1863, M&B 2050, SC-2159, and M&B 1950. There was no cross-tolerance between SC-1718 and C6. Although mice treated with C6 developed a considerable degree of tolerance to TEAB, mice treated with daily injections of TEAB did not develop tolerance to C6.

Tolerance to the Action of Methonium Compounds on Isolated Intestinal Preparations

The smallest dose which would abolish the contractions of the circular muscle of isolated strips of ileum taken from normal guinea-pigs and rabbits was determined for various drugs. For a 100 ml. organ-bath, the doses were as follows: C6 (0.3–2.0 mg.), M&B 2050 (0.2–0.5 mg.), M&B 1863 (0.3–2.0 mg.), M&B 1950 (0.5–2.0 mg.), M&B 2024B (0.5–2.0 mg.), TEAB (2–5 mg.), and TMAB (4–6 mg.). TMAB is tetramethylammonium bromide, and M&B 2024B is hexamethylene 1:6-bis-*N*-(*N*-methyl-pyrrolidinium) dibromide. A typical result is illustrated in Fig. 4a. This shows that, although a small dose of C6 is sufficient to abolish the contractions of circular muscle, a period of washing is followed by complete recovery.

By contrast, intestinal strips obtained from animals pre-treated with C6 consistently showed a very high degree of tolerance to C6 (Fig. 4b). The pre-treatment consisted in giving daily subcutaneous injections increased gradually from 20 mg. to 50 mg. for rabbits and from 5 mg. to 15 mg. for guinea-pigs over a 2-week period. Under these conditions, such high concentrations of C6 as 100 mg./100 ml. produced neither abolition of the contractions of the circular muscle nor the appearance of any latent period. Furthermore, there was a very high degree of tolerance to M&B 1863, M&B

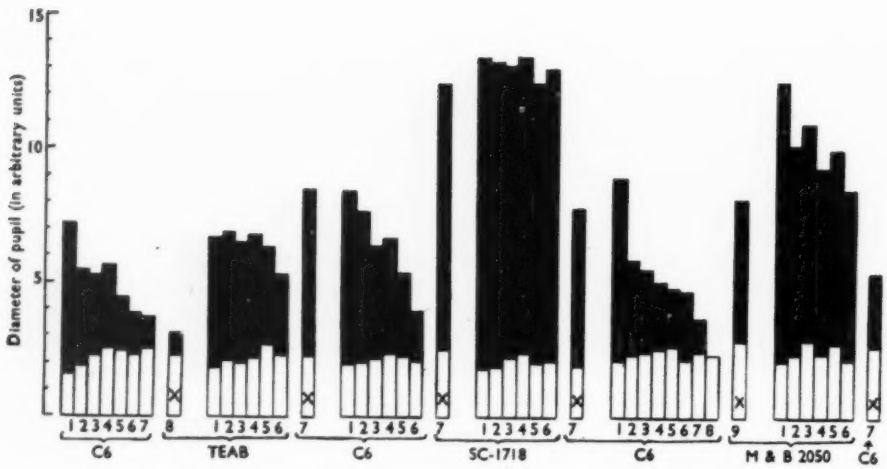

FIG. 2.—Histograms showing the progressive development of tolerance and cross-tolerance to C6 and related drugs. The numbers under each column refer to the day of injection. An X shows the result of a cross-over test, e.g., the effect of the standard dose of TEAB on mice already treated with C6. The whole column gives the mean diameter of the pupil after the injection of the drug (15 mice). The blank part gives the mean initial diameter and the solid part the mean increase in pupil diameter. These results are representative.

1950, M&B 2050 and M&B 2024B. Concentrations of the order of 100 mg./100 ml. of M&B 1950 or M&B 2050 were unable to prevent the contractions of the circular muscle obtained from C6-treated animals. Pre-treatment with C6 also established a high degree of tolerance to TEAB and a considerable degree of tolerance to TMAB. Thus with TMAB a dose of 30 mg. had no effect, though a 60 mg. dose in the 100 ml. bath abolished the contractions (compared with a 4–6 mg. dose for an intestinal strip from an untreated animal). Yet intestinal preparations taken from TEAB-treated animals showed no cross-tolerance to C6, the dose of C6 necessary to abolish the contractions in such preparations being of the same order as for untreated animals (about 2 mg./100 ml.). These preparations, however, exhibited a considerable degree of tolerance to TEAB. It should be added that small doses of TEAB produced immediate contraction of the longitudinal muscle of the TEAB-tolerant ileum, spontaneous contractions of this muscle not normally being observed with preparations from guinea-pigs. Subsequent elevation of the intraluminal pressure gave rise to considerable spasm of the circular muscle, but the addition of further quantities of TEAB (up to 30–45 mg./ml.) was followed by paralysis of the circular muscle.

In a further series of experiments, intestinal strips obtained from untreated guinea-pigs and

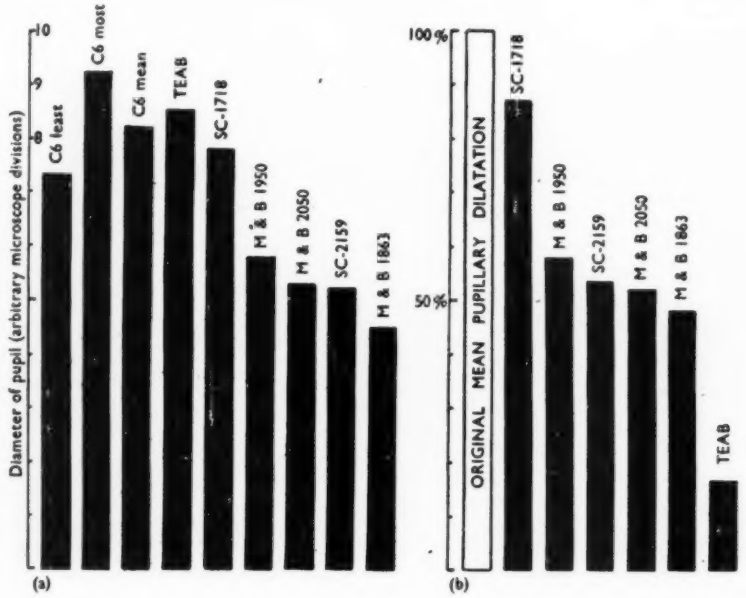

FIG. 3.—Histograms showing the degree of cross-tolerance obtained with the repeated daily injection of C6 and related compounds. The cross-over was performed after one of the pair of drugs had been injected daily for 6–8 days. Each column gives the mean effect for 15 mice. (*a*) The first three columns represent the mean pupillary dilatation in separate experiments with C6. The remaining columns show the mean pupillary dilatation following the test dose of C6 in groups of mice previously treated with the methonium compound named above each column. (*b*) The drugs named above each column were given after C6. The height of each column gives the degree of cross-tolerance as a percentage calculated by comparing the mean effect of the test dose after C6 with its effect in untreated mice.

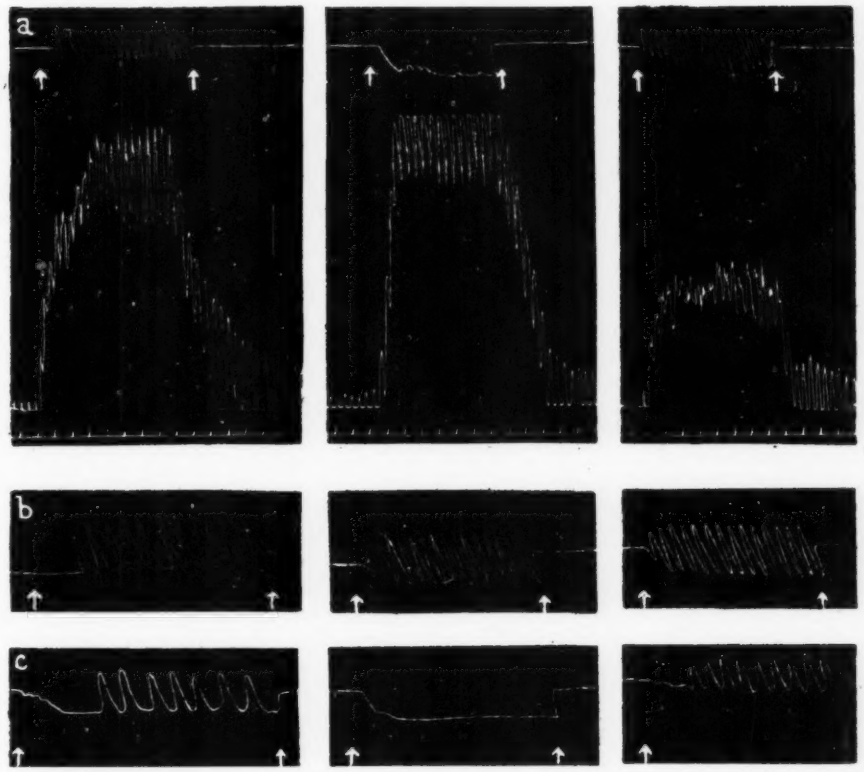

FIG. 4.—Trendelenburg preparations of rabbit ileum. Between the arrows, the intraluminal pressure was raised by 2.5 cm. of water. The effect of this on the circular muscle is shown on all records and the effect on the longitudinal muscle on the top set of records. (*a*) The first record shows a control contraction, the second the abolition of the reflex by hexamethonium bromide (2 mg./100 ml. organ-bath), and the third the restoration of the reflex after washing. (*b*) The ileal strip used in this experiment was taken from a C6-tolerant rabbit. The first record shows a control contraction. The second record shows a contraction after 5 mg. C6, and the third after 75 mg. C6 had been added to the bath. (*c*) Normal rabbit's ileum pre-treated *in vitro* for 3 hr. with 1 μg./ml. of C6 in Ringer-Tyrode. The first record shows a control contraction after washing, the second the effect of C6 (1 mg./100 ml.), and the third the effect obtained after the C6 had been washed out of the organ-bath.

rabbits were kept in Ringer-Tyrode solution containing C6 (1 mg./ml.) at room temperature for 2–4 hr. before being used for Trendelenburg preparations. After repeated washing of the strip, the dose of C6, or of another methonium compound, needed to abolish the peristaltic reflex was determined and compared with that required for untreated intestinal strips. A typical result is shown in Fig. 4c. Here a 1 mg. dose of C6 in the 100 ml. bath was sufficient to abolish the reflex contractions of a strip even after treatment *in vitro* for 3 hr. with C6-containing Ringer-Tyrode solution. Tolerance does not seem to have developed. Related compounds, tested on other preparations that had been treated *in vitro* with C6, gave similar results ; the doses of these methonium compounds which abolished the circular muscle contractions of the treated strips were similar to the doses which abolished the circular muscle contractions of the untreated strips.

Tolerance to the Action of Methonium Compounds on the Superior Cervical Ganglion of the Cat

The development of tolerance after a single intravenous dose of C6 (5 mg./kg.) was studied in 6 cats. The drug was injected while the nictitating membrane was being kept contracted by stimulating the peripheral end of the pre-ganglionic cervical sympathetic nerve with spiked electrical shocks applied at the rate of 10/sec. When a second, equal dose of C6 was injected several hours later, it produced less blocking of the superior cervical ganglion (as judged from the contractions of the nictitating membrane) and a smaller fall of blood pressure. Such experiments indicate that in *intact* cats tolerance to the ganglion-blocking action of C6 develops even after a single intravenous dose. Tolerance could not be demonstrated within 2 hr. of an injection, but it was demonstrable within 3–4 hr. ; after 24 hr. it was well marked (Fig. 5).

Vascularly Isolated Ganglion Preparations.—The first part of the next series of experiments was as above: after an initial control contraction had

FIG. 5.—Cat. Pentobarbitone-urethane anaesthesia. Response of nictitating membrane to pre-ganglionic stimulation. The white line marks the period of stimulation. An arrow indicates the intravenous injection of C6 (5 mg./kg.). (*a*) Control contraction. (*b*) Block produced by first dose of C6. (*c*) Control contraction 24 hr. later. (*d*) Partial ganglionic blockade produced by second dose of C6. The smaller effect of C6 indicates the development of tolerance.

been recorded, an intravenous dose of C6 was given while another contraction was being recorded ; then, 24 hr. later, another control contraction and the effect of a second intravenous dose of C6 were recorded. Next, 3–4 hr. later, after the superior cervical ganglion had been isolated from the rest of the body but for its vascular and nervous connexions, it was perfused with a blood substitute. The third dose of C6 was injected into the perfusion cannula.

Three such experiments were performed ; Fig. 6 shows a typical result. The initial dose of C6 produced almost complete ganglionic blockade, as indicated by the near absence of contraction of the nictitating membrane ; but there was recovery after 24 hr. and the second dose did not produce such powerful blocking. The intra-arterial injection of C6 3–4 hr. later produced very little ganglionic blockade. Therefore, provided that the dose given intra-arterially (0.1–0.5 mg. of C6 according to the size of the animal) gives a concentration of C6 in the blood reaching the ganglion comparable to that obtained after the intravenous injections, it would seem that the tolerance to C6 which has developed in the intact animal remains, and can be exhibited in the isolated sympathetic ganglion preparation. Perfusion of the ganglion with a blood substitute—regarded as equivalent to washing the ganglion under pressure—did not remove the tolerance. In a further 2 experiments, the intra-arterial dose of C6 was increased to 1 mg. and was given immediately before the contraction of the nictitating membrane was elicited by stimulating the preganglionic nerve. The results of these 2 experiments were similar to those of the 3 preceding.

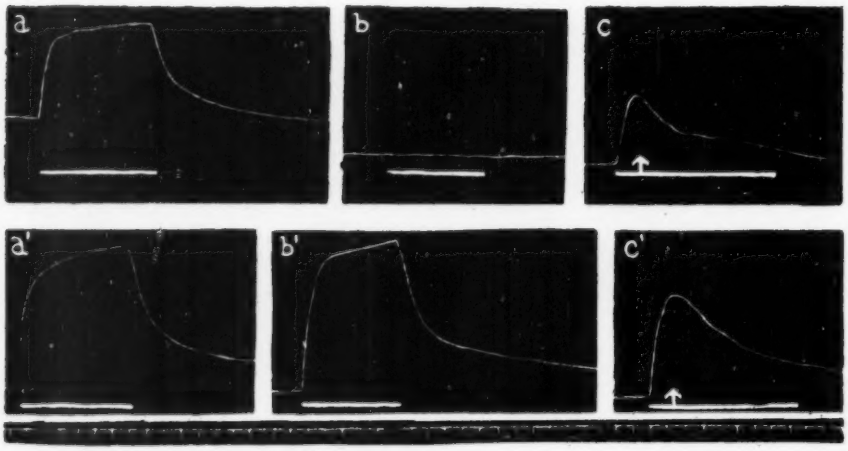

FIG. 6.—Cat. Pentobarbitone-urethane anaesthesia. The contractions of the right and left nictitating membranes (a,b,c and a′,b′,c′ respectively) induced by stimulation of both pre-ganglionic cervical sympathetic nerves have been recorded simultaneously. The white lines mark the periods of stimulation, and the arrows the intravenous injection of C6 (5 mg./kg.). Time, 10 sec. (*a*, *a′*) Control contractions of nictitating membranes. (*b*) After 20 min. application of 1% C6 to the right superior cervical ganglion. (*b′*) After 20 min. application of isotonic saline to the left superior cervical ganglion. (*c*, *c′*) Records obtained simultaneously 24 hr. later. Meanwhile the animal had been continuously anaesthetized, the right ganglion treated with 1% C6 solution and the left ganglion with isotonic saline except during the last half-hour, during which period both ganglia were washed with normal saline.

Local Application of C6.—Fig. 6 illustrates the result of an experiment in which both ganglia were exposed and isolated but for their nervous and vascular connexions, after which one ganglion was treated for 24 hr. with C6 and the other with saline. The initial control contractions were unequal, although the conditions on both sides seemed to be identical as regards both the balancing and magnification of the levers and the state of the membranes and ganglia. In 4 other experiments performed under the same conditions unequal contractions of the two nictitating membranes were also obtained. No explanation for the inequality could be found. After an intravenous injection of C6, the percentage reduction in the size of the contractions was the same for the treated and untreated sides. Thus, there seemed to be no development of tolerance on the treated side although the ganglion had been surrounded by pledgets of cotton-wool soaked in 1% C6 solution for 24 hr. This suggests that the local application of C6 to ganglia is not sufficient to give rise to tolerance. It is possible, however, that these results might be explained by the unphysiological conditions of the experiment. It was therefore decided to perfuse the ganglion with the blood substitute previously used, since it was thought that perfusion under pressure might bring the C6 into more intimate contact with the ganglion.

The first set of experiments, performed on 3 cats, differed from the preceding set in that the ganglion on one side was treated with C6 by perfusing it for 1 hr. with blood substitute containing 5 μg./ml. of C6 and then for another 30–60 min. with the simple blood substitute. A subsequent test dose of C6 produced much the same response as the initial test dose, indicating that tolerance had not occurred. The same result was obtained in the following 6 experiments: 3 differed from the last

only in that heparinized blood from untreated patients was used in place of the blood substitute; in 1 heparinized blood from an untreated cat was used in place of the blood substitute; and in 2 the C6 was given by injecting 5 mg. of the bromide dissolved in 5 ml. of 0.9% NaCl slowly and continuously into the perfusion cannula over a period of 15 min. by means of an electrically driven syringe, the blood substitute being perfused at the same time. It was always found that while the C6 solution was being perfused the contractions of the nictitating membrane in response to preganglionic stimulation were completely abolished; but subsequent perfusion with the blood substitute alone or with heparinized blood that did not contain C6 was followed by complete recovery of the ganglion sensitivity to C6 and to other methonium compounds.

Attempted Reversal of Tolerance by Local Washing.—The general plan of the next series of experiments was (i) to make the whole animal tolerant to C6, and then (ii) to wash one superior cervical ganglion repeatedly to see whether this procedure could restore the sensitivity to C6 of the washed ganglion, even though the rest of the animal remained tolerant as shown by the reaction of the other superior cervical ganglion and nictitating membrane.

In 3 experiments, cats were made tolerant to C6 by giving two 5 mg./kg. doses, the first 24 hr., and the second 20 hr., before the washing. Next, the right superior cervical ganglion was dissected and isolated from the rest of the body except for its nervous and vascular connexions. Contractions of the nictitating membranes were recorded at this stage, as before and after the test doses of C6. Finally, the right ganglion was washed externally with saline for 30–60 min., a third test dose of C6 (5 mg./kg.) was given intravenously, and contractions of both nictitating membranes were recorded. With each cat it was found that both ganglia exhibited approximately equal tolerance to the test doses of C6; the sensitivity of the washed ganglion was reduced to about the same extent as that of the control. In 2 of these experiments the post-ganglionic nerve was stimulated after a further dose of C6. This produced as big a contraction as the control stimulation, indicating that the block had occurred at the ganglion.

As it seemed possible that washing the ganglion from the outside with saline might not be adequate to remove a tolerance-producing substance, a further 3 experiments were performed in which the right common carotid artery was cannulated and the corresponding ganglion was perfused at a pressure of 140–160 mm. Hg for 1 hr. with the plain blood substitute, after control measurements had been made as before. Here, too, tolerance was unaffected by the washing, for the injection into the perfusion cannula of a 1 mg. dose of C6—calculated to be equivalent to a body dose of at least 5 mg./kg.—produced no greater degree of ganglionic blockade than was observed with the control ganglion after an intravenous dose of 5 mg./kg. of C6.

Effects of Blood or Plasma from C6-tolerant Animals or Patients.—The evidence already given suggested that, although tolerance to C6 develops at autonomic ganglia and cannot be removed by washing, it cannot be produced by applying C6 directly to an isolated ganglion. Presumably the ganglion is affected by a process that occurs elsewhere in the body. It therefore appeared of interest to see if an isolated ganglion could be made tolerant to C6 by treating it with blood obtained from C6-tolerant animals, for tolerance could be explained by the elaboration in one part of the body of a substance which in one way or another neutralized the effect of C6.

Single vascularly isolated ganglion preparations were used for the remaining 11 experiments. They were perfused initially with the blood substitute. During this part of the experiment the effects of various test doses of C6 injected at the height of contraction of the nictitating membrane were recorded. At least 15 min. rest period was allowed between two successive recordings and the volume of all injections into the perfusion cannula was kept constant. Next, the perfusion medium was changed for 15–60 min. to the heparinized whole blood of cats treated previously with C6 (5 experiments) or to blood substitute containing plasma obtained from hypertensive patients under treatment with C6 (6 experiments). Lastly, the simple blood substitute was perfused again while C6 was injected at intervals in the test doses used previously.

The result of a typical experiment is shown in Fig. 7. During the first period of perfusion with the blood substitute no tolerance developed at the ganglion. After perfusion of the ganglion with the blood or plasma obtained from the C6-tolerant cats or patients, tolerance now developed at the ganglion. The second series of test doses of C6 produced much smaller effects than the control injections, even no effect on occasion. In addition, cross-tolerance to other methonium compounds developed. Evidently the blood of C6-tolerant patients or animals can confer on autonomic ganglia the property of tolerance to C6 and related

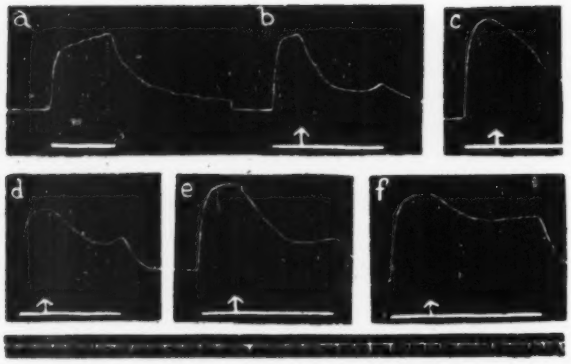

FIG. 7.—Cat. Pentobarbitone-urethane anaesthesia. Perfused superior cervical ganglion-nictitating membrane preparation. Contractions of nictitating membrane obtained by pre-ganglionic stimulation. The white lines show periods of stimulation, the arrows the injection of various C6 solutions into the perfusion cannula. Time, 10 sec. (*a*) Control contraction. (*b*) Effect of C6 (30 μg.) in 2 ml. of saline. (*c*) C6 (20 μg.) in 2 ml. of saline. (*d*) C6 (30 μg.) in 2 ml. of blood plasma of a C6-tolerant patient. (*e*) C6 (100 μg.) in 2 ml. of blood plasma of a C6-tolerant patient. (*f*) After 15 min. perfusion of the ganglion with blood substitute containing 1 part in 4 of plasma from a C6-tolerant patient. C6 (200 μg.) in 2 ml. of plasma injected at the arrow.

drugs. When such tolerance was produced, it it could not be removed by local washing or by perfusion under pressure for 1–2 hr.

DISCUSSION

The experiments on mice show that when hexamethonium (C6) is given repeatedly, considerable tolerance to its mydriatic action develops. However, all the actions of C6 are not affected to the same extent, for it has been found that the LD50 was not raised to a significant extent by the prior administration of substantial doses of C6. The signs shown by the dying animals suggest that C6 killed mainly—if not entirely—by a curariform action. Whatever the action of C6 that causes death in mice, little or no tolerance develops to it.

Tolerance has been demonstrated for four actions of C6, all of which depend on its ability to block autonomic ganglia—mydriasis, impairment of the peristaltic reflex, lowering of blood pressure, and reduction of the response of the nictitating membrane to stimulation of the cervical sympathetic chain. There seems little doubt when all four actions are considered that tolerance to them represents a change in the sensitivity of the ganglion rather than, say, a compensatory cardiovascular mechanism of the type suggested by Paton and Zaimis (1952) and Morrison (1953).

The rate of development of tolerance and cross-tolerance in the intact animal seems to be similar to that observed clinically (Smirk and Alstad, 1951 ; Smirk, 1952, 1953). Tolerance seems to develop slowly. It reaches a maximum in weeks rather than days and, when this point has been reached, the degree of tolerance remains more or less constant provided that the administration of the drug is continued. The development of tolerance is not only slow and progressive but appears to be preceded by a lag period. In the cat, tolerance could not be demonstrated until at least 3 hr. had elapsed after an intravenous dose of C6. Similarly, although tolerance could be demonstrated in mice after 24 hr., it was much more noticeable at the end of a week.

The impression was gained from animal experiments that the degree of development of tolerance is related to the size and frequency of the dose. So long as the dose is not sufficiently large to injure the animal, the development of tolerance could be hastened by increasing the size and frequency of the dose. This observation is consistent with the clinical observations of Freis (1951), who thought that the development of tolerance to C6 was delayed by giving injections at intervals of 12 hr. instead of more frequently. Similar phenomena have been noted by Smirk (personal communication). In animals as in man, tolerance and cross-tolerance to methonium compounds wear off after the treatment has been stopped for about two weeks.

The results of experiments performed with isolated ganglion preparations taken from tolerant animals indicate that tolerance and cross-tolerance can be demonstrated in preparations containing either parasympathetic ganglia (intestinal strips) or sympathetic ganglia (nictitating membrane preparation). The tolerance and cross-tolerance could not be reversed by washing the isolated preparation, even by washing under pressure as in the perfusion of an isolated superior cervical ganglion for periods of up to 2 hr.

By contrast, the local action of C6 applied to or perfused through isolated ganglion preparations did not give rise to any tolerance or cross-tolerance. This happened also when the superior cervical ganglion was perfused with homologous animal or human blood to which C6 was added after bleeding, indicating that the presence of normal blood does not play a part in the development of tolerance at the isolated ganglion. When, however, the ganglion was perfused with the blood of animals or patients previously treated with C6, tolerance rapidly developed and, as with the isolated ganglion preparations obtained from tolerant animals, it could not be removed by washing or by perfusion for periods of up to 2 hr.

It seems reasonable to conclude that in the intact animal, or in man, the development of tolerance and cross-tolerance is not a local reaction of autonomic ganglia to the presence of C6, but that

the administration of C6 to the whole animal is followed after an interval of a few hours by the appearance in the blood of a substance which reduces ganglionic sensitivity to C6 and related compounds. No similar substances seems to be present in the blood of untreated patients or animals. Evidence as to the site of formation of this substance will be presented in a later paper.

Summary

1. Tolerance to the ganglion-blocking action of hexamethonium and related substances follows their administration to intact laboratory animals. However, the LD50 of hexamethonium for mice was not raised significantly by the prior repeated administration of substantial doses of the drug. Death in mice from hexamethonium is attributed mainly to a curariform as distinct from a ganglion-blocking action.

2. Tolerance can be demonstrated in isolated ganglion preparations obtained from animals previously treated with hexamethonium.

3. The local application of hexamethonium to isolated ganglion preparations from untreated animals does not give rise to tolerance.

4. The application of blood from tolerant patients or animals, to isolated ganglion preparations from untreated animals, is followed by the development of tolerance at the ganglia.

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THE ACTION OF LOCAL ANAESTHETICS ON EXPERIMENTAL EPILEPSY IN CATS AND MONKEYS

BY

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(RECEIVED FEBRUARY 24, 1955)

Very little attention has been paid to the action of local anaesthetics on the functions of the central nervous system. In the current literature there is no mention of their striking effect, which will be dealt with in this paper, on experimental epileptic attacks and related central phenomena. A thorough search of the literature revealed two papers by Mercier (1950a and b) on the effect of different local anaesthetic drugs on audiogenic seizures in albino rats given subconvulsive doses of various convulsants. As far as we know no attention has been paid to these investigations, which seem to be the first to indicate that local anaesthetics, such as procaine, have an anticonvulsant effect.

We have studied the effects of different local anaesthetics on the electrically evoked epileptiform cortical after-discharge and related corticospinal phenomena, and compared them with the effects of these drugs on spinal functions and ascending transmission mechanisms. Preliminary reports on the central effects of lidocaine have already appeared (Bernhard and Bohm, 1954c and d). We have also compared the antiepileptic effect of these substances and barbiturates and of combinations of barbiturates and local anaesthetics. These investigations form the basis for the therapeutic use of a local anaesthetic, lidocaine (lignocaine A.N., "xylocaine"), for the rapid abolition of severe grand mal attacks and of post-operative epileptic seizures (Bernhard, Bohm, and Höjeberg, 1955).

METHODS

Cats and monkeys were used. Recordings of the epileptiform cortical after-discharge following repetitive cortical stimulation were made from the exposed brain. It is well known that the duration of the post-stimulatory epileptiform cortical after-discharge depends on such stimulation characteristics as total duration, frequency, and strength (see, for example, Noel, 1941; Walker and Johnson, 1948; Bernhard, Bohm, and Taverner, 1954), and on the narcotic level, as well as on the state of the cortex with reference to a preceding cortical stimulation (see, for example, Dusser de Barenne and McCulloch, 1937, 1939; Bernhard *et al.*, 1954). The type of cortical after-discharge and of the accompanying motoneurone activity, which in the non-curarized state results in epileptiform muscular convulsions, also varies with these different factors. Based on our recent studies of experimental epilepsy (Bernhard *et al.*, 1954), we used stimuli of about 1 msec. at 25/sec. for a total duration of 5 sec. The corticogram was recorded throughout the experiment, and cortical stimulation was not repeated until the corticographic changes from the preceding stimulation had disappeared. Usually there was a pause of 3–4 min. between each series of cortical stimulations. Pentobarbitone sodium, usually 15 mg./kg., was injected before the operation (opening of the skull, dissection of nerves, and sometimes laminectomy). Often 5 mg./kg. pentobarbitone was given after operation and before the stimulation, (+)-tubocurarine was added to prevent disturbing muscular contractions during the experimental epileptic attack. The narcotic level may have been different in the different experiments—an important point in the interpretation of the effects of the local anaesthetics. We have, therefore, made a closer study of the summation of the barbiturates and the local anaesthetics (see p. 293). Under conditions fixed with respect to the different factors mentioned, the duration of the epileptiform cortical after-discharge was surprisingly constant during a long period. In several experiments the action potentials were also led off from different nerves and ventral roots during and after repetitive cortical stimulation. In one series of experiments the mono- and polysynaptic reflex responses in the lumbar ventral roots to electrical stimulation of different nerves and dorsal roots were recorded. Finally, the response within the receiving area II of the cortex to afferent stimulation was led off with the same type of electrodes as those used for the corticograms (chlorided silver electrodes with a diameter of 0.5 mm.).

RESULTS

General Effect of a Local Anaesthetic on the Post-stimulatory Epileptiform Cortical After-discharge

Fig. 1 shows the corticograms (cat) from the frontal (tracing 1) and parietal (tracing 2) areas

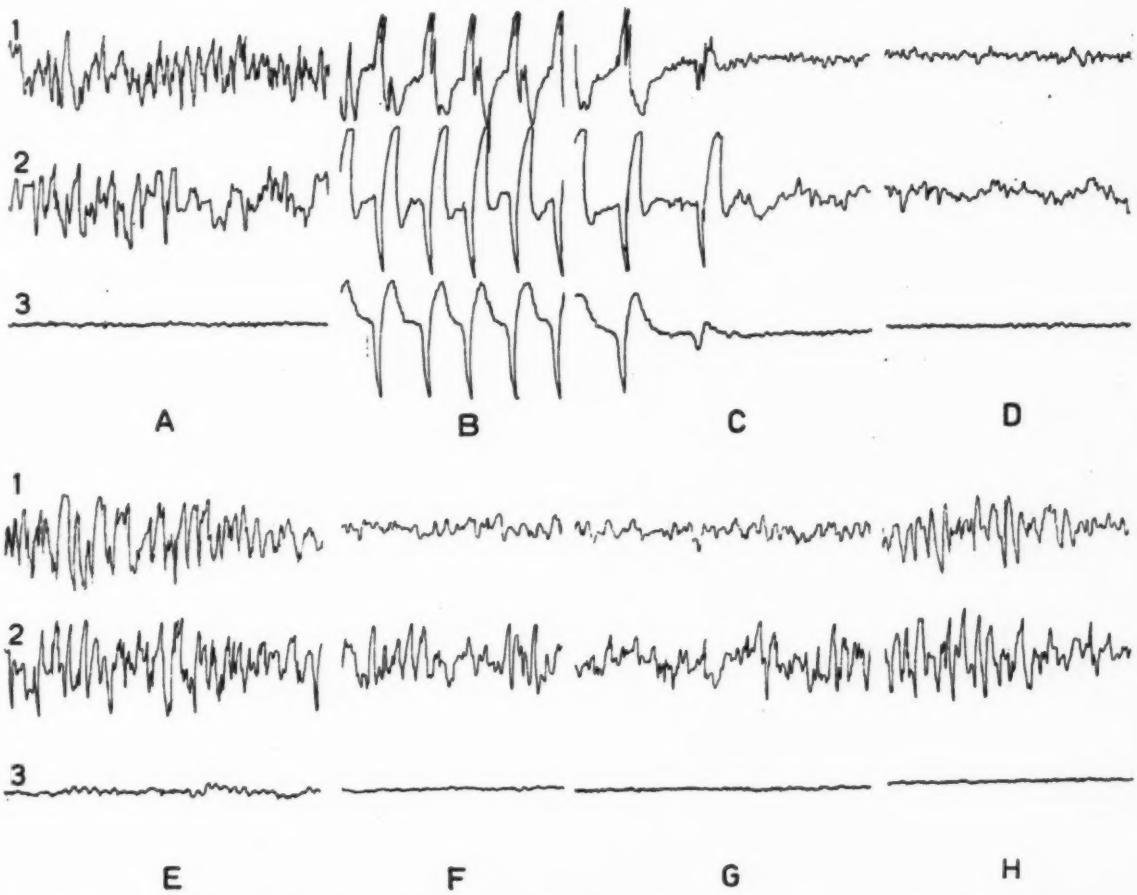

FIG. 1.—Cortical activity led off from the frontal (tracing 1) and parietal (tracing 2) areas in cat before (A–D) and 5 min. after (E–H) intravenous injection of 2 mg./kg. lidocaine. A and E before stimulation; B and F 5 sec., C and G 15 sec., D and H 25 sec. after cessation of repetitive cortical stimulation (25/sec., for 5 sec.) of the ipsilateral sensorimotor area.

ipsilateral to the stimulating electrodes placed within the precentral region. Tracing 3 is from the L7 ventral root contralateral to the cortical area stimulated. Repetitive cortical stimulation elicited a typical epileptiform after-discharge (Fig. 1 B–C). The hypersynchronous cortical potentials as well as the rhythmical outbursts of "clonic" type in the L7 ventral root continued for 12 sec. after the cessation of the repetitive stimulation, and their frequency decreased in a typical way before they finally disappeared (Fig. 1 C). This convulsive attack was followed by a diminution ("exhaustion") of the cortical potentials (Fig. 1 C and D). An intravenous injection of lidocaine (2 mg./kg. in 1% w/v solution) was then given, and 5 min. later the cortex was again stimulated. Records F–H in Fig. 1 show that the injection of the lidocaine was followed by a total abolition of the poststimulatory epileptiform attack and that the cortical "exhaustion" was less pronounced than before the injection (*cf.* Fig. 1 D and H), whereas it had no significant effect on the prestimulatory corticogram (*cf.* Fig. 1 A and E).

The recovery of the poststimulatory after-discharge was investigated by testing the effect of cortical stimulation at various intervals after the injection. The injection of 2 mg./kg. lidocaine usually caused a total abolition of the cortical after-discharge for 10–15 min. followed by recovery to the pre-injection value in about 60 min. (filled circles in Fig. 2). A smaller dose (1 mg./kg.), however, generally did not produce a complete block but reduced the duration of the poststimulatory epileptiform attack to 30–50% of the pre-injection value (open circles in Fig. 2). Fig. 2 also shows the surprising constancy, in the absence of a drug, of the duration of the cortical after-discharge for 60 min. (crosses)—a necessary condition for our test experiments.

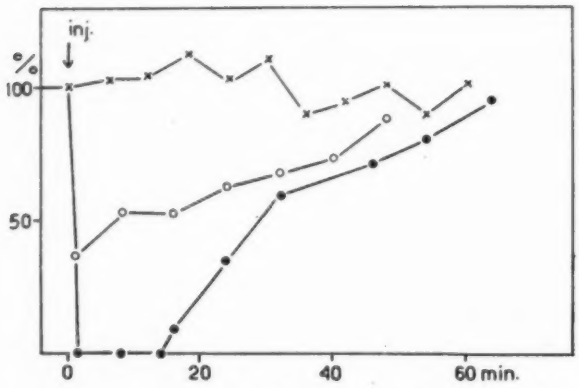

FIG. 2.—Duration of cortical after-discharge as a percentage of pre-injection values (vertical axis) plotted against time (horizontal axis) after intravenous injection of 1 mg./kg. (open circles) and 2 mg./kg. (filled circles) of lidocaine. The crosses represent individual duration values of cortical after-discharge obtained during 60 min. before injections. The filled circles are from the experiment in Fig. 1.

It should be mentioned that slow intravenous injections of lidocaine, in the doses used in these experiments, are known not to influence the blood pressure (Goldberg, 1949) ; we also confirmed this.

Comparison of Different Local Anaesthetics on the Poststimulatory Cortical After-discharge

Different intravenous doses (0.5–4 mg./kg.) of procaine, butethamine (*iso*butylaminoethyl *p*-aminobenzoate, " monocaine "), lidocaine (diethylaminoacetat-2 : 6-xylidide), diethoxine (diethylaminoethyl 4-ethoxylbenzoate hydrochloride), and tetracaine (2-dimethylaminoethanol 4-*n*-butylaminobenzoate hydrochloride, amethocaine) were tested in cats and monkeys. All of them, depending upon the dose, blocked or diminished the epileptiform after-discharge. The curves in Fig. 3 illustrate the protective action against experimental epileptic fits of 2 mg./kg. of the drugs mentioned. Fig. 3 gives the results from a series of experiments performed on the same preparation under similar conditions. As seen, lidocaine was more effective than procaine and butethamine. The two latter substances had about the same effect on the cortical after-discharge. Diethoxine, however, was of about the same potency as lidocaine. Tetracaine, finally, always gave a more prolonged effect than lidocaine. To evaluate the effect of these substances on the cortical after-discharge, several series of experiments were performed, the drugs being given in such amounts that they only produced a partial block (see curves for procaine and butethamine in Fig. 1). For example, in one series of experiments the doses of the different local anaesthetics which gave a reduction of the duration of the cortical after-discharge to 50% were found to be 3 mg./kg. for procaine, 2 mg./kg. for butethamine, 1 mg./kg. for lidocaine and diethoxine, and 0.2 mg./kg. for tetracaine. The experiments referred to were all performed on cats. Our experiments on the antiepileptic effects of the different local anaesthetics in monkeys gave similar results.

One of the main reasons for these introductory investigations was to find which substances of this type would be most convenient as anticonvulsants in certain clinical conditions (*cf.* Bernhard, Bohm, and Höjeberg, 1955). As shown above, tetracaine was the most efficient, but it would not be convenient for clinical intravenous use because of its relatively high toxicity. Of the others lidocaine and diethoxine were the most potent. Since lidocaine seems to be less toxic than diethoxine and since, in addition, there is considerable evidence that the intravenous injection of lidocaine in man carries little risk of complications (Clive-Lowe, Gray, Spencer and North, 1954), lidocaine was the chief drug used in our further investigations on the central effects of local anaesthetics.

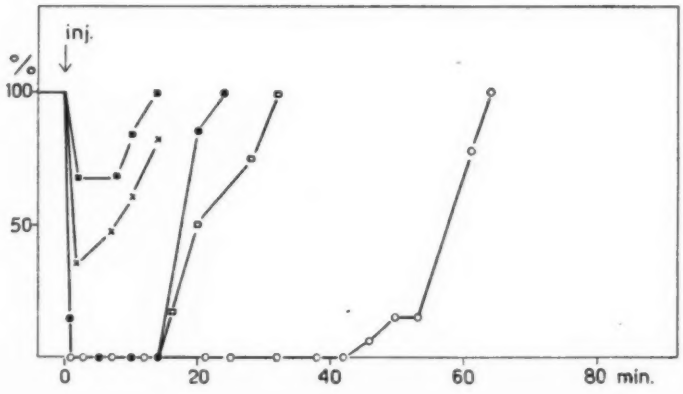

FIG. 3.—Duration of cortical after-discharge as a percentage of pre-injection values (vertical axis) plotted against time (horizontal axis) after intravenous injection of 2 mg./kg. of different local anaesthetics. Filled rectangles, procaine; crosses, butethamine; filled circles, lidocaine; open rectangles, diethoxine; open circles, tetracaine.

The Antiepileptic Effect of Lidocaine Compared with its Effect on Other Central Functions

So far we have only discussed the duration of the after-discharge, since this characteristic of the epileptiform attack seemed to be valuable for the study of anticonvulsants on this specific central phenomenon (see Bernhard *et al.*, 1954). The epileptiform motoneurone activity evoked by electrical cortical stimulation often shows a " tonic-clonic " phase followed by a " clonic " phase (see Rosenblueth, Bond, and Cannon, 1942 ; Jasper, 1954 ; Bernhard *et al.*, 1954) the electrical signs of which can be recorded from the ventral root. In Fig. 4, obtained from an experiment on a monkey, the heavy line at the top marks the total duration of the cortical after-discharge (12 sec.) following cessation of repetitive cortical stimulation (zero at the horizontal axis), and the thin line immediately beneath it marks the duration of the " tonic " component of the poststimulatory L7 ventral root activity. During the first period of the poststimulatory phase the segmental monosynaptic reflex was greatly facilitated. In earlier investigations we have shown how this facilitatory action on the motoneurones is built up during repetitive cortical stimulation, and how the facilitation continues after cortical stimulation has stopped (Bernhard and Bohm, 1954b). Fig. 5 shows the monosynaptic reflex in L7 ventral root elicited by stimulation of the biceps nerve before (A) and after (B) repetitive cortical stimulation. The monosynaptic reflex, tested at different intervals after cessation of cortical stimulation, was facilitated (Fig. 4, dashed curve) for 3–4 sec. after cessation of stimulation, that is for about the same time as the " tonic after-discharge " (thin horizontal line). The facilitation was followed by a depression of the monosynaptic reflex for about 20 sec. (*cf.* Bernhard *et al.*, 1954). After the injection of 2 mg./kg. lidocaine the cortically evoked poststimulatory facilitation of the monosynaptic reflex disappeared entirely (Fig. 5 C and D). Fig. 4 shows the amplitude changes of the monosynaptic reflex during the poststimulatory period 3 (a), 8 (b), and 14 (c) min. after the injection of lidocaine. The amplitude of the monosynaptic reflex was decreased during the poststimulatory period after the lidocaine, which in this experiment blocked the poststimulatory after-discharge for 14 min.

This experiment shows that the cortically evoked poststimulatory facilitation of the monosynaptic

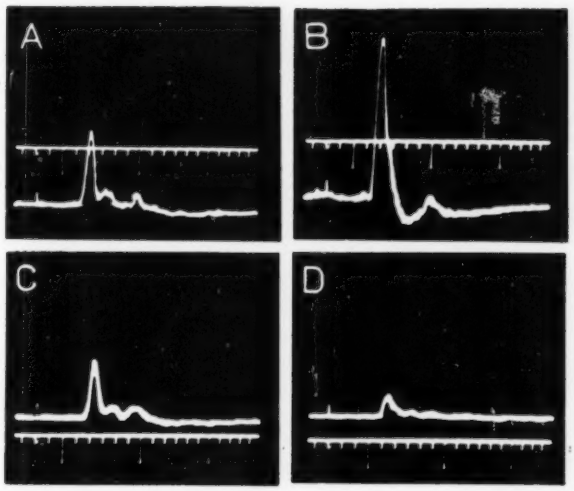

FIG. 5.—Monosynaptic reflex response in L7 ventral root following stimulation of the ipsilateral biceps nerve in monkey. A, before cortical stimulation; B, 2 sec. after conditioning repetitive cortical stimulation (25/sec., for 5 sec.) of the contralateral precentral region. C and D, the same after 3 mg./kg. lidocaine i.v.

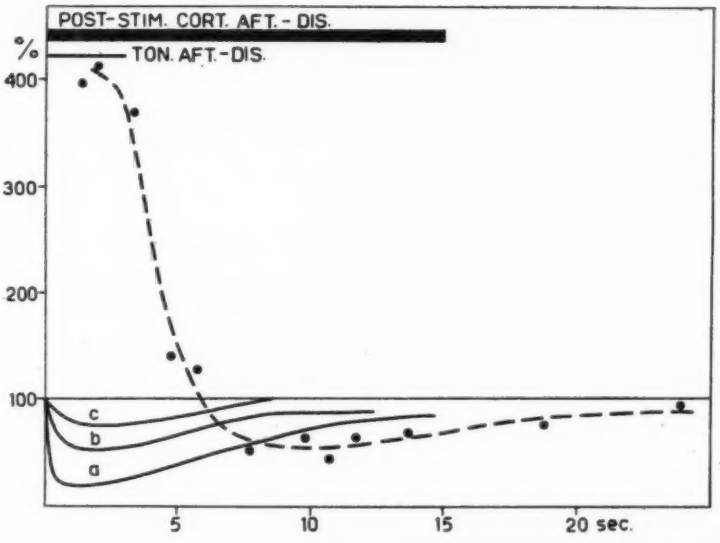

FIG. 4.—Amplitude of the monosynaptic reflex response in the L7 ventral root to stimulation of the ipsilateral biceps nerve during the period following the cessation (zero on horizontal axis) of conditioning repetitive cortical stimulation (25/sec., for 5 sec.) of the contralateral precentral area. The amplitude values are given as a percentage of the unconditioned values obtained before cortical stimulation (100% on vertical axis). Dashed curve shows the poststimulatory facilitation and subsequent depression of the monosynaptic reflex before the injection of 3 mg./kg. lidocaine. Curves a, b, and c (unbroken) show the poststimulatory depression of the monosynaptic reflex after the injection of 3 mg./kg. lidocaine. Heavy line marks the duration of the poststimulatory cortical after-discharge and the thin line the duration of the tonic phase of the poststimulatory ventral root activity before the injection of lidocaine. Monkey.

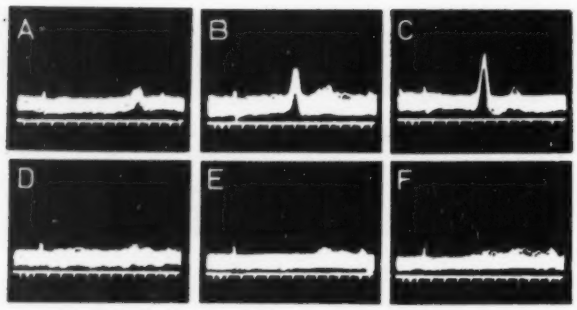

FIG. 6.—Superimposed action potentials in the left radial nerve following each stimulus in a train of shocks (25/sec.) delivered to the fore limb subdivision of the right precentral cortical area 1 (A and D), 3 (B and E), and 5 (C and F) sec. after the beginning of the repetitive stimulation, before (A–C) and 3 min. after intravenous injection of 4 mg./kg. lidocaine (D–F). Time in msec. Monkey.

reflex disappears in parallel with the abolition of the epileptiform attack.

The response in different nerves or ventral roots to each cortical stimulus in a train of repetitive shocks consists of a series of action potentials with different latencies. Fig. 6 A–C shows a series of superimposed records of the action potentials in the radial nerve of a monkey following each cortical stimulus in a train of shocks (25/sec.) at different intervals after the beginning of the repetitive stimulation. In our earlier investigations evidence was presented that in *monkeys* the earliest response which is built up during the repetitive cortical stimulation is monosynaptically transmitted from the corticospinal neurones to the motoneurones (Bernhard, Bohm, and Petersén, 1953; Bernhard and Bohm, 1954a and b). The building up of this monosynaptic response during the course of the repetitive cortical stimulation is shown in Fig. 7 A (filled circles). According to the earlier investigations mentioned, the increase of the monosynaptic response to cortical stimulation reflects the building up of cortically evoked facilitatory effect, the poststimulatory phase of which is represented by the dashed curve in Fig. 4.

It was therefore of interest to investigate the effect of the local anaesthetics on the building up of the monosynaptic motoneurone response to cortical stimulation. Records 6 D–F were taken 3 min. after injecting lidocaine (4 mg./kg.) at the same intervals during the repetitive cortical stimulation as records 6 A–C which were taken before injecting lidocaine. No monosynaptic response was built up during the stimulation period used (open circles in Fig. 7 A). Fig. 7 B shows that the cortical after-discharge evoked by the repetitive stimulation was also totally abolished 3 min. after the injection of lidocaine. Six min. after the injection (crosses in Fig. 7 A) the monosynaptic response to each cortical shock slowly built up to the original value and the cortical after-discharge had recovered to 40% of the pre-injection value (see Fig. 7 B). Finally, 11 min. after the injection, the building up of the monosynaptic response (rectangles in Fig. 7 A) was normal, and the cortical after-discharge had recovered (see Fig. 7 B).

In contrast to the very striking blocking effect on the poststimulatory epileptiform attack, as well as on the corticospinal facilitatory action during the repetitive cortical stimulation, the same doses of local anaesthetics had comparatively little influence on certain other central functions. In cats the effects of lidocaine on the amplitude of the monosynaptic and polysynaptic reflexes were tested as well as its effects on the cortical area II responses to electrical stimulation of low threshold fibres in cutaneous nerves. The effect on the cortical after-discharge to cortical stimulation was tested in the same experiments. Typical results of such an experiment are shown in Fig. 8. The reflex responses were reduced to 70–90% of the pre-injection values, during the period when the cortical after-discharge was totally abolished by lidocaine and there was no effect at all on the amplitude of the cortical response to the ascending volley elicited by stimulation of low threshold cutaneous afferents. In other experiments of this type, and in experiments on non-anaesthetized spinal preparations, the amplitudes of the reflex responses were only slightly reduced or unaltered by the injection of a similar dose of lidocaine.

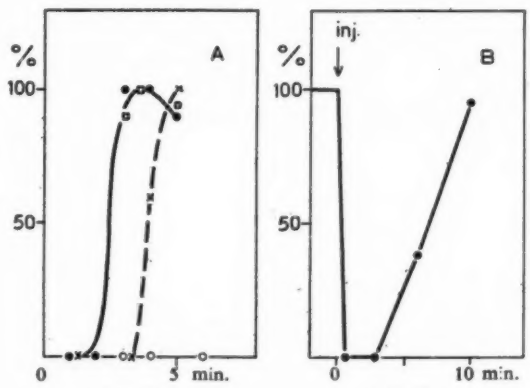

FIG. 7A

FIG. 7B

FIG. 7A.—Amplitude of the monosynaptic response in the radial nerve to contralateral cortical stimulation at different intervals after the beginning of the repetitive cortical stimulation as a percentage of the highest value obtained. Filled circles (unbroken curve) before lidocaine; open circles 3 min., crosses (dashed curve) 6 min., after 2 mg./kg. lidocaine i.v.

FIG. 7B.—Duration of the poststimulatory cortical after-discharge as a percentage of pre-injection values plotted against time after the injection of 2 mg./kg. lidocaine. Monkey.

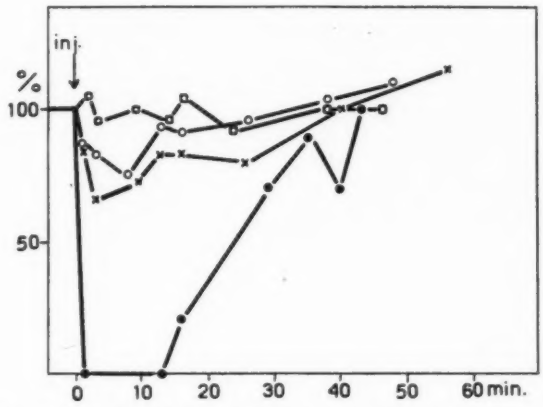

FIG. 8.—Effects of 2 mg./kg. lidocaine i.v. on the duration of the cortical after-discharge (filled circles), monosynaptic reflex (crosses), polysynaptic reflex (open circles) and sensory area II response to stimulation of low threshold afferent fibres (rectangles). The values for the duration of the cortical after-discharge as well as for the amplitude of the potential responses are plotted as a percentage of the pre-injection values against time after the injection.

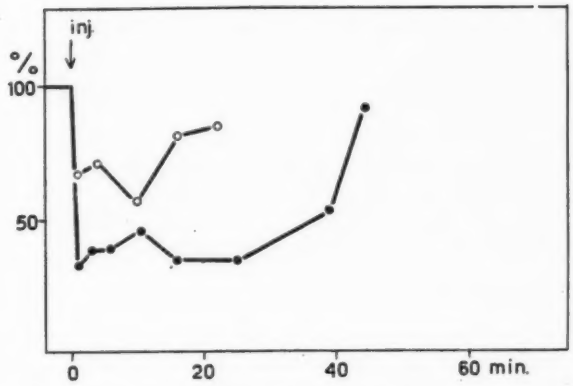

FIG. 9.—Duration of cortical after-discharge as a percentage of the pre-injection values plotted against time after the injection of 1 mg./kg. lidocaine (open circles) and 3 mg./kg. phenobarbitone (filled circles).

Comparison of the Action of Lidocaine and Barbiturates, and Summation of Their Effects, on the Cortical After-discharge

The effect of phenobarbitone (3 mg./kg.) on the cortical after-discharge was compared with that of lidocaine (1 mg./kg.) in a cat under light pentobarbitone anaesthesia (Fig. 9). The experiment shows that—in such a preparation at least—lidocaine is more effective in blocking the cortical after-discharge than is phenobarbitone.

Since, as mentioned, all our experiments were on cats and monkeys under pentobarbitone, and since the barbiturates themselves influence the cortical after-discharge, it was of importance to obtain information about the summation of the effects of pentobarbitone and a local anaesthetic on the cortical after-discharge. This is shown in Fig. 10 A and B for pentobarbitone and lidocaine in a monkey. A small dose of pentobarbitone (15 mg./kg.) was given before opening the skull. The average duration of the cortical after-discharge was then estimated. The average pre-injection value of the duration of the cortical after-discharge was 12 sec. Lidocaine (1 mg./kg.; arrow 1 in Fig. 10 A), in this lightly anaesthetized preparation, did not have any significant effect on the duration of the cortical after-discharge. Thirteen min. after the lidocaine, pentobarbitone (1 mg./kg.) was injected; this dose did not have any effect on the cortical after-discharge either (arrow 2 in Fig. 10 A). Eight min. later a further lidocaine injection (1 mg./kg.; arrow 3 in Fig. 10 A) was followed by a dramatic effect on the cortical after-discharge, its duration being reduced to about 20% of the pre-injection value. Experiments of this type show

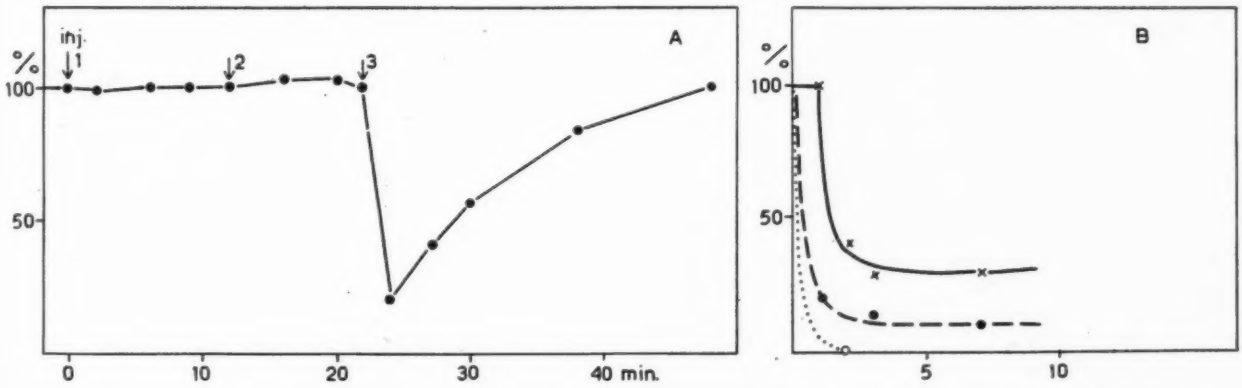

FIG. 10.—Summation effect of lidocaine and pentobarbitone on the poststimulatory cortical after-discharge. (A) Duration of cortical after-discharge as a percentage of pre-injection values plotted against time. Arrows 1 and 3 mark injections of 1 mg./kg. lidocaine, and arrow 2 marks the injection of 1 mg./kg. pentobarbitone. (B) Crosses show the duration of cortical after-discharge as a percentage of pre-injection values plotted against the amount of pentobarbitone injected. Circles show the duration of the cortical after-discharge as a percentage of the pre-injection values after the injection of 1 mg./kg. (filled circles) and 2 mg./kg. (open circles) lidocaine plotted against dose of pre-injected pentobarbitone (mg./kg.).

that pentobarbitone in small doses increases the sensitivity of the cortical after-discharge to the local anaesthetic.

Fig. 10 B, obtained from the same experiment, shows how, up to a certain point, the injection of increasing doses of pentobarbitone increased the sensitivity to lidocaine (1 mg./kg.). The effect on the duration of the cortical after-discharge of different doses of pentobarbitone alone is included: at 1 mg./kg. there was no effect, but 2 mg./kg. reduced the duration of the after-discharge to 40%. It is of interest that a further increase (up to 7 mg./kg.) only caused a reduction of the duration of the after-discharge to about 30% ; it was never abolished. Lidocaine (1 mg./kg.) had no influence on the cortical after-discharge before, but reduced it to 20% after, the pentobarbitone ; the pentobarbitone alone had no effect. Larger doses of pentobarbitone increased the effect of lidocaine (1 mg./kg.) only slightly. If, instead, 2 mg./kg. of lidocaine was injected after 1 mg./kg. of pentobarbitone, the after-discharge was totally abolished (dotted curve in Fig. 10 B). Some important general implications of these results will be taken up in the discussion. Here it should only be stressed that the amount of pre-injected pentobarbitone, up to a certain dose, modifies the sensitivity of the cortical after-discharge to local anaesthetics. Because of this fact we tried to keep the different preparations on the same narcotic level when testing the effect of the different local anaesthetics on the cortical after-discharge.

Discussion

It is perhaps not surprising that there has been relatively little mention in the current literature of the effects of local anaesthetics on the central nervous system, because several central functions —for example, the spinal reflexes and the transmission in the ascending system connected to low threshold afferent fibres—were found to be very little affected by intravenous injections of local anaesthetics in moderate doses (see Fig. 8). This is in striking contrast to the pronounced blocking effect of the same doses of the drugs on the poststimulatory epileptiform cortical after-discharge. Further investigations are being performed in order to find the mode and site of action of these drugs in the complex mechanisms—known to be both cortical and subcortical—involved in the epileptiform attack (see, for example, Jasper, 1954). The facts that the pre-stimulatory corticograms, as well as the cortical response within the cortical receiving area II, are not significantly altered, indicate that the excitability of at least part of the cortical neurones is not affected by doses which produce a total abolition of the poststimulatory after-discharge. It is interesting to note that the facilitatory effect on the motoneurones built up by cortical stimulations (Figs. 6 and 7), which persists after the cessation of the repetitive cortical stimulation (Figs. 4 and 5), is abolished by lidocaine in parallel with the abolition of the cortical after-discharge. There are reasons to assume that this effect is owing to an action on the transmission systems mediated over the bulbar reticular formation, the functions of which have been thoroughly investigated by Magoun and his colleagues (Magoun, 1950), but we have not tested this assumption experimentally.

It should also be mentioned that the blocking effect of the local anaesthetics on the cortical after-discharge offers a means of studying the different steps in the elicitation of experimental epilepsy. For instance, during the period when the cortical after-discharge is abolished after the injection of lidocaine, some of the post-stimulatory effects on different spinal functions may still be present in the absence of the cortical after-discharge (Fig. 4, curves a–c). Thus different kinds of corticospinal activity may continue as the result of the cortical stimulation by itself and not as a result of the epileptiform attack—in contrast to certain forms of corticospinal activity which are due to the poststimulatory epileptic fit.

Among the different local anaesthetics tested tetracaine was found to have a more pronounced protective effect against the poststimulatory cortical after-discharge than lidocaine and diethoxine, these two substances being more effective than butethamine and procaine. The order of effectiveness of these substrates as local anaesthetics (see, for example, Wiedling, 1953 ; Gray and Geddes, 1954) seems to be the same as that of their effectiveness as anticonvulsants. In this connexion, however, it should be mentioned that other substances which are active as local anaesthetics had an opposite effect on the duration of the cortical after-discharge: it is thus not possible to conclude that there is a direct relationship between local anaesthetic activity as such, and anticonvulsant activity.

Our study of the summation effect of a local anaesthetic and a barbiturate shows (1) that the administration of pentobarbitone alone reduces the duration of the cortical after-discharge, but, even in relatively big doses, does not abolish it ; (2) that small doses of pentobarbitone increase the sensitivity of the cortical after-discharge to lidocaine, but that this effect reaches a maximum after quite small doses of pentobarbitone ; (3) that only a small increase of the lidocaine dose is followed

by a total abolition of the cortical after-discharge in preparations which have received a small dose of pentobarbitone.

The effect of the local anaesthetics against experimental epileptic attacks seems to us to be quite striking. Since, however, the experiments were performed on animals under light pentobarbitone anaesthesia they do not prove the effectiveness in the absence of barbiturates. However, we have already found that moderate doses of intravenously injected lidocaine stop status epilepticus in man not given any other anticonvulsant drugs (Bernhard, Bohm, and Höjeberg, 1955). The reasons for using lidocaine in most of these studies, which serve as a basis for the clinical application, have already been mentioned. It should only be added that the results of the experiments on the summation effects of lidocaine and pentobarbitone have been confirmed in investigations in man (Bernhard, Bohm, Höjeberg, and Melin, 1955). The conclusion is that a combination of a small dose of barbiturate with lidocaine is the most effective treatment for the temporary abolition of severe epileptic fits. In this way, also, excitatory effects of lidocaine, which have very rarely been observed after injection in man (Clive-Lowe, Gray, Spencer, and North, 1954), are avoided.

Summary

1. The effects of local anaesthetics (procaine, butethamine, lidocaine, diethoxine, and tetracaine) on experimental epilepsy, as well as on other types of central activity, are described in cats and monkeys—under light pentobarbitone anaesthesia.

2. Intravenous injections of these local anaesthetics reduce or abolish the poststimulatory epileptiform cortical after-discharge elicited by cortical stimulation, without altering the prestimulatory EEG. The effect depends on the dose and on the degree of anaesthesia. Tetracaine is more effective than lidocaine and diethoxine, and lidocaine and diethoxine are more effective than procaine and butethamine. Taking into account the relation between the toxicity of these substances and their effectiveness against experimental epilepsy, lidocaine appears to be the most suitable anticonvulsant for clinical use.

3. Lidocaine, in doses which block the epileptiform cortical after-discharge, abolishes the cortically evoked facilitatory effect on the spinal motoneurones which is built up during the repetitive cortical stimulation. In connexion with these findings some aspects of experimental epilepsy are discussed.

4. Lidocaine, in doses which abolish the experimental epileptic fit, has no significant effect on the spinal reflexes; the monosynaptic and polysynaptic reflexes may be reduced only to 70–90% of the pre-injection values. Finally, the cortical response within the sensory area II to ascending volleys evoked by stimulation of low threshold cutaneous fibres is not influenced at all by lidocaine in doses which abolish the experimental epileptic attack.

5. The experiments on the summation effects of a barbiturate (pentobarbitone) and lidocaine show (1) that pentobarbitone alone, in suitable doses, reduces the duration of the epileptic fit; (2) that the injection of pentobarbitone in doses too small to affect the epileptic fit, increases the anti-epileptic effect of lidocaine; (3) that even 2 mg./kg. lidocaine abolishes the epileptic fit in preparations which have received a small dose of pentobarbitone (20–25 mg./kg.).

6. We conclude that a combination of a small dose of a barbiturate and a moderate dose of lidocaine should be used for the temporary abolition of epileptic fits in man.

This work has been supported by grants from Magnus Bergvalls Stiftelse and AB.Astra, Södertälje.

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NOMOGRAMS FOR MULTIPLE SLOPE-RATIO ASSAYS ARRANGED IN BLOCKS, AND IMPROVED NOMOGRAMS FOR ASSAYS WITHOUT BLOCKS

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(RECEIVED MARCH 9, 1955)

It is commonly advantageous with multiple assays, which involve several test preparations at a time, to incorporate in the design some form of local control, usually by blocking in complete replications. In a microbiological assay, for example, blocks might consist of different racks or different days. The ordinary methods of analysis can of course be used in such cases, but repeated applications become tedious, particularly for slope-ratio assays, in which the responses are approximately linearly related to the doses. It is the intention of this paper to present graphical methods for the rapid analysis of results of multiple slope-ratio assays arranged in blocks, and tables will be given from which the nomogram for a particular design may easily be drawn up.

The methods have been developed in general terms, but only the results for the most usual designs will be given here. Attention will thus be restricted to assays in which there are two non-zero doses of each preparation, with a common zero dose, and with the number of replications for each dose-level of the standard preparation and for the zero dose greater by a factor of approximately $\sqrt{(v-1)}$ than the number of replications for the other treatments, where v is the number of preparations, including the standard. The reduction of the general method for cases when there is no blocking results in simpler methods than those previously presented (Clarke and Hosking, 1953) both for multiple assays and for simple assays, that is, assays of only one test preparation at a time, and the results for simple assays will also be described here. Numerical examples will illustrate the construction and use of the nomograms.

Estimation of Error Variance.—For slope-ratio assays arranged in blocks the appropriate estimate of error variance is given, as well as by the within-block replication mean square, by the interaction of treatments with blocks after the elimination of the interaction with blocks of the multiple regression on doses (Clarke, 1955). Hence the error variance estimate may be derived from the interactions of blocks with what have been described as " blanks " and " intersections " (Finney, 1951; Clarke, 1952).

This method of estimating the error variance has been used in deriving the nomograms presented here for multiple assays arranged in blocks; as usual for mainly graphical methods, the estimates are made from ranges so as to shorten the calculations. Assays which are not arranged in blocks afford an easy estimate of the appropriate error variance from the ranges of observations on the same dose-level and preparation.

NUMERICAL EXAMPLES

Multiple Slope-ratio Assay Arranged in Randomized Blocks

The data used in this example are taken from the results of a microbiological assay of cyanocobalamin in samples of cows' milk, taken at different stages of lactation. The complete experiment involved 16 milk samples and a standard, each at three equally spaced dose-levels, with a common zero dose, but it is sufficient to consider here only six of the samples and the two lower dose-levels, and in each block two tubes for each dose-level of the standard and for the zero dose. A complete replication of the tests was carried out on each of two days, so that there were two blocks. The results of the assay and the numerical computations required are presented in Table I.

The upper dose of each preparation is taken to be 1 unit, so that with equal spacing the lower dose is then $\frac{1}{2}$ unit. The individual results are listed as shown in the first two rows (a and b), and totals are calculated where there are replicates within a block, as for the standard and zero dose. The remaining calculations are as follows.

1. Block differences (Block 1—Block 2), for each column.
2. $\delta_H = 2\{$value in column (c)$\} - \{$value in column (d)$\}$, calculated from block differences, for each preparation.
3. r = range of values of δ_H, using *mean* for standard preparation.
4. Block totals (Block 1+Block 2), for each column.
5. $H = 2\{$value in column (c)$\} - \{$value in column (d)$\}$, calculated from block totals, for each preparation.
6. Q, calculated for each preparation as shown underneath Table I.
7. $S(Q)$ = total of values of Q.
8. $S(Q)/Q$, for each preparation (a slide-rule gives sufficient accuracy).

Thus for sample 1, for example

$$\delta_H = (2\times 15) - 12 = 18,$$
$$H = (2\times 71) - 110 = 32,$$
$$Q = 18\{71 + (2\times 110)\} - 3690 = 1548,$$
$$S(Q)/Q = 5526/1548 = 3.57.$$

Before proceeding to estimate relative potencies, tests of the validity of the assay are made, and the form of these tests in this particular example is found from the lower part of Table III. Corresponding to the test for " blanks " (Clarke, 1952) we have the criterion (i) that $S(H) - 4T_0$ should not be greater than $3.47r$. The coefficient of r here depends on the critical probability level to be used: in Table III the values appropriate to a 5% level have been presented. The test for " intersections " (Clarke, 1952) is conveniently split up into two parts: (ii) $S(H) - 4H_S$ should not be greater than $4.47r$, and (iii) range (H_T), the range of the values of H for the test preparations only, should not be greater than $2.14r$. As may be seen from the bottom of Table I, the tests for validity are satisfied in this example.

It is now possible to proceed immediately to estimate the relative potencies and their fiducial limits from Fig. 1. The linear parts of the diagram are substantially the same as the preliminary chart described in the earlier paper (Clarke and Hosking, 1953), but use of the exact fiducial limits enables curves for finding the limits to be superimposed on the same chart, and the limits are found with only one alignment.

The value of b_S, the slope of the dose-response line for the standard preparation, is first found by joining $S(Q)/Q_S = 1.71$ on AA′ to $S(Q) = 5526$ on BB′, and reading off $b_S = 49$ on CC′, whence b_S/r is calculated as 1.88. The point corresponding to $S(Q)/Q_S$ on AA′ is joined to the point corresponding to $S(Q)/Q_T$ on DD′, for each test preparation in

TABLE I

COMPUTING SHEET FOR A MULTIPLE SLOPE-RATIO ASSAY

The responses are readings on a photo-electric absorptiometer, multiplied by 10

Preparation	Control	Standard		Sample 1	Sample 2	Sample 3	Sample 4	Sample 5	Sample 6	Total	Range
Dose (Units)	0	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)	½ (c)	1 (d)		
Responses:																			
Block 1 (a)	7 9	35 38	63 66																
	16	73	129	43	61	37	62	25	44	19	35	23	43	33	54	697			
Block 2 (b)	10 9	25 30	51 51																
	19	55	102	28	49	30	51	19	31	17	23	17	26	24	42	533			
Block differences:																			
(a)−(b)	−3	18	27	15	12	7	11	6	13	2	12	6	17	9	12	164			
$\delta_H = 2$(c)−(d)		9 (mean = 4·5)		18		3		−1		−8		−5		6				26 = r	
Block totals:																			
(a)+(b)	T_0 35	S_1 128	S_2 231	T_{11} 71	T_{12} 110	T_{21} 67	T_{22} 113	T_{31} 44	T_{32} 75	T_{41} 36	T_{42} 58	T_{51} 40	T_{52} 69	T_{61} 57	T_{62} 96	1,230 = G			
$H = 2S_1 - S_2$		25		32		21		13		14		11		18		134 = $S(H)$			
Q		3,240		1,548		1,584		−198		−954		−486		792		5,526 = $S(Q)$			
$S(Q)/Q$		1·71		3·57		3·67		−27·9		−5·80		−11·37		6·98					
From chart:																			
Relative potency				0·98		0·99		0·58		0·41		0·52		0·81					
5% fiducial limits				0·87		0·88		0·48		0·30		0·41		0·70					
				1·11		1·12		0·70		0·52		0·63		0·93					
Calculated values:																			
Relative potency				0·984		0·992		0·585		0·413		0·520		0·811					
5% fiducial limits				0·882		0·890		0·489		0·315		0·423		0·713					
				1·092		1·101		0·682		0·509		0·616		0·913					

$Q_S = 18(S_1 + 2S_2) - 6G$; $Q_{Tt} = 18(T_{t1} + 2T_{t2}) - 3G$; $b_S = 49$; $b_S/r = 1{\cdot}88$

$6G = 7{,}380$ $\quad 3G = 3{,}690$

Validity tests: $S(H) - 4T_0 = -6$; $t_1 r = 90$; $S(H) - 4H_S = 34$; $t_2 r = 116$; Range (H_T) = 21; $t_3 r = 56$ } (5% probability level)

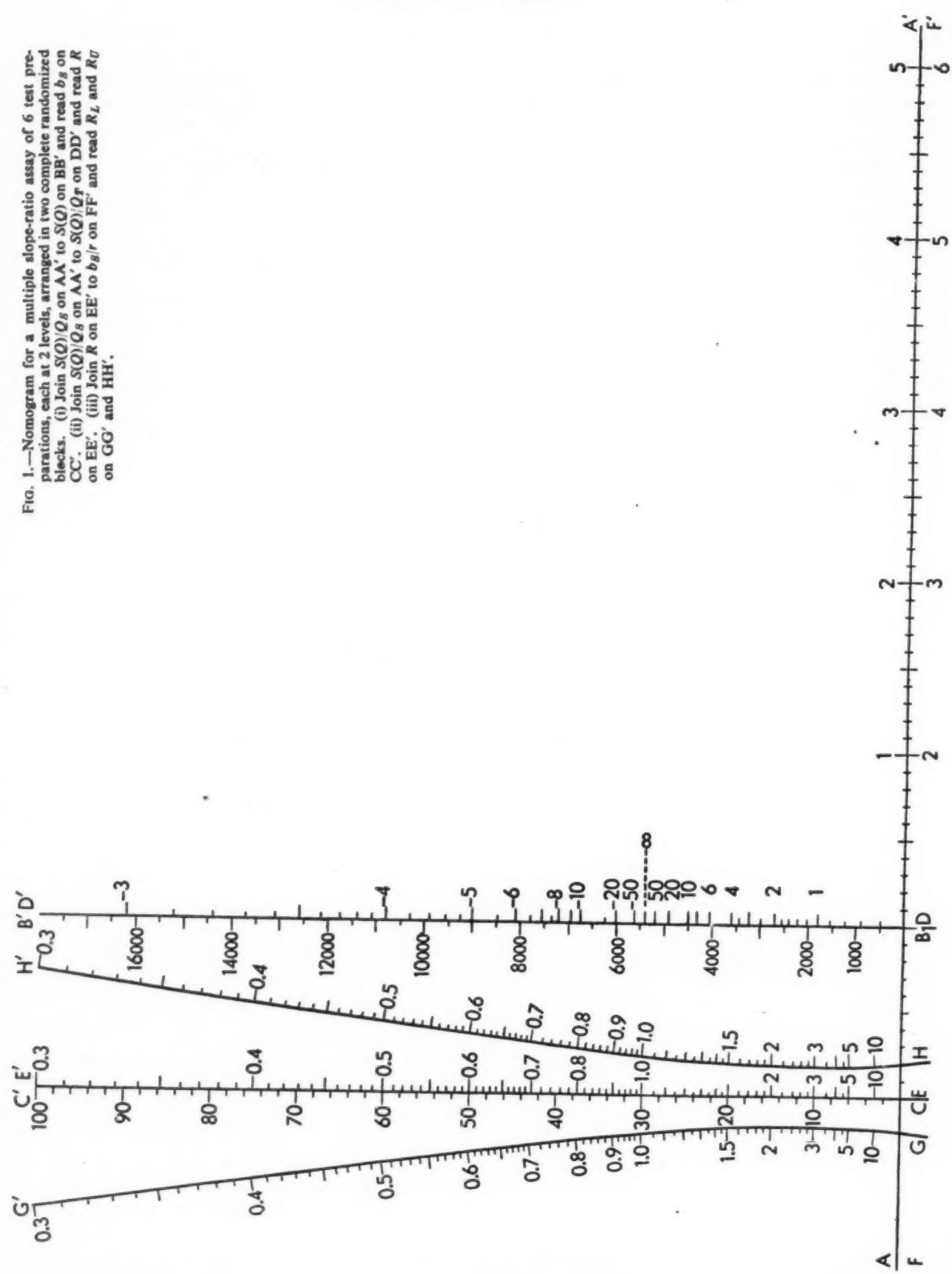

FIG. 1.—Nomogram for a multiple slope-ratio assay of 6 test preparations, each at 2 levels, arranged in two complete randomized blocks. (i) Join $S(Q)/Q_S$ on AA' to $S(Q)$ on BB' and read b_S on CC'. (ii) Join $S(Q)/Q_S$ on AA' to $S(Q)/Q_T$ on DD' and read R on EE'. (iii) Join R on EE' to b_S/r on FF' and read R_L and R_U on GG' and HH'.

turn, and the relative potency (R) is read off on EE′. This point on EE′ is then joined to the point for b_S/r on FF′; the intersections with the curves GG′ and HH′ give the lower and upper 5% fiducial limits. The vertical scales for these curves are the same as for the line EE′. Thus for sample 1, when the point $S(Q)/Q_S=1.71$ is joined to the point $S(Q)/Q_T=3.57$ on DD′, the relative potency R is found from EE′ to be 0.98, and joining this point on EE′ to the point $b_S/r=1.88$ on FF′ the intersections with GG′ and HH′ give 5% fiducial limits of 0.87 and 1.11. The calculated values of the relative potencies and their fiducial limits are given for comparison in Table I, and it may be seen that the approximate estimates of the limits obtained by using the range estimate of error are within 0.02 of the calculated values. The fact that the approximate fiducial interval is wider than the calculated interval is due to the discrepancy between the range estimate of error variance (9.99) and the mean square estimate (7.85).

Simple Slope-ratio Assay, without Blocks

Table II shows the results and computations for another assay of cyanocobalamin, this time a simple assay of only one milk sample, with a common zero dose and two non-zero doses of each preparation. There were four replications in this 5-point assay, and there were no blocks in the design.

TABLE II

COMPUTING SHEET FOR A SIMPLE SLOPE-RATIO ASSAY

Preparation	Control	Standard		Test		Total
Dose (units)	0	½	1	½	1	
Response ..	0·8	3·9	7·0	2·3	3·9	
	0·8	3·8	6·8	2·6	4·0	
	0·9	3·7	7·2	2·2	4·0	
	1·0	3·8	6·6	2·2	4·2	
	T_0	S_1	S_2	T_1	T_2	
Total ..	3·5	15·2	27·6	9·3	16·1	$71·7=G$
Range ..	0·2	0·2	0·6	0·4	0·3	$1·7=r$

Validity tests

$H=2S_1-S_2$, etc.	2·8	2·5	$5·3=S(H)$
$S(H)-2T_0=-1·7$; $t_1r=2·60$ / $H_S-H_T=0·3$; $t_2r=2·21$			(5% probability level)

Estimation of relative potency and fiducial limits

$Q=5(S_1+2S_2)-3G$, etc.	136·9	−7·6
Q_T/Q_S	—	−0·0555
Q_S/r	80·5	—
From chart:		
R (relative potency) ..	—	0·52
5% fiducial limits ..	—	0·49 0·55
Calculated values:		
R	—	0·523
5% fiducial limits ..	—	0·492 0·555

The preliminary calculations required are indicated in the table; they are the same as those given in the earlier paper (Clarke and Hosking, 1953). The estimate of error is obtained from the sum (r) of the ranges for each treatment, and validity tests are made as described in the 1953 paper. The change to be described here is in the form of the chart, which is illustrated in Fig. 2. This, besides giving the exact fiducial limits instead of the approximate ones, is more convenient for use than the chart published earlier, since the upper and lower fiducial limits are found with one alignment. It is also possible to design one chart for any number of replications, provided the number of doses of each preparation remains constant.

In this example, since $S(H)-2T_0$ is not greater than t_1r and H_S-H_T is not greater than t_2r, where t_1 and t_2 are found, from the bottom of Table V, to have the values 1.53 and 1.30 respectively, the validity tests are satisfied, and we may proceed to estimate the relative potency and its fiducial limits. The relative potency, R, is read off immediately on the opposite side of the scale (AA′) for Q_T/Q_S: the value, −0.0555, of Q_T/Q_S, corresponds to a relative potency of 0.52. To find the fiducial limits, a line through the point corresponding to $Q_S/r=80.5$ on CC′ is produced through the point for $n=4$ (n is the number of replications) on DD′ to meet EE′ at a point X, say. This point X is then joined to the point corresponding to $Q_T/Q_S=-0.0555$ on AA′ and the 5% fiducial limits, 0.49 and 0.55, are given by the intersections with the curves FF′ and GG′. The scales for these curves are linear in the vertical direction, and are the same as that for R. The scale marks on EE′ are needed only for the construction of the chart.

The range estimate of error variance in this example (0.0272) is very close to the direct estimate (0.0263), and consequently the approximate fiducial interval agrees well with the calculated interval.

If n is unaltered from assay to assay, it may be more convenient to draw up a chart for the fixed value of n. This is done as explained in the later section on the construction of the chart: briefly, the scales CC′ and DD′ are omitted, and a scale for Q_S/r is made on the line EE′.

METHOD OF CONSTRUCTION OF CHARTS

Nomograms suitable for a wide range of the most usual types of assay designs may be easily constructed using the coordinates and scale factors presented in Tables III, IV, and V. The tables have been drawn up so as to give charts fitting a sheet of paper 20 units high and 30 units wide. Adjustment

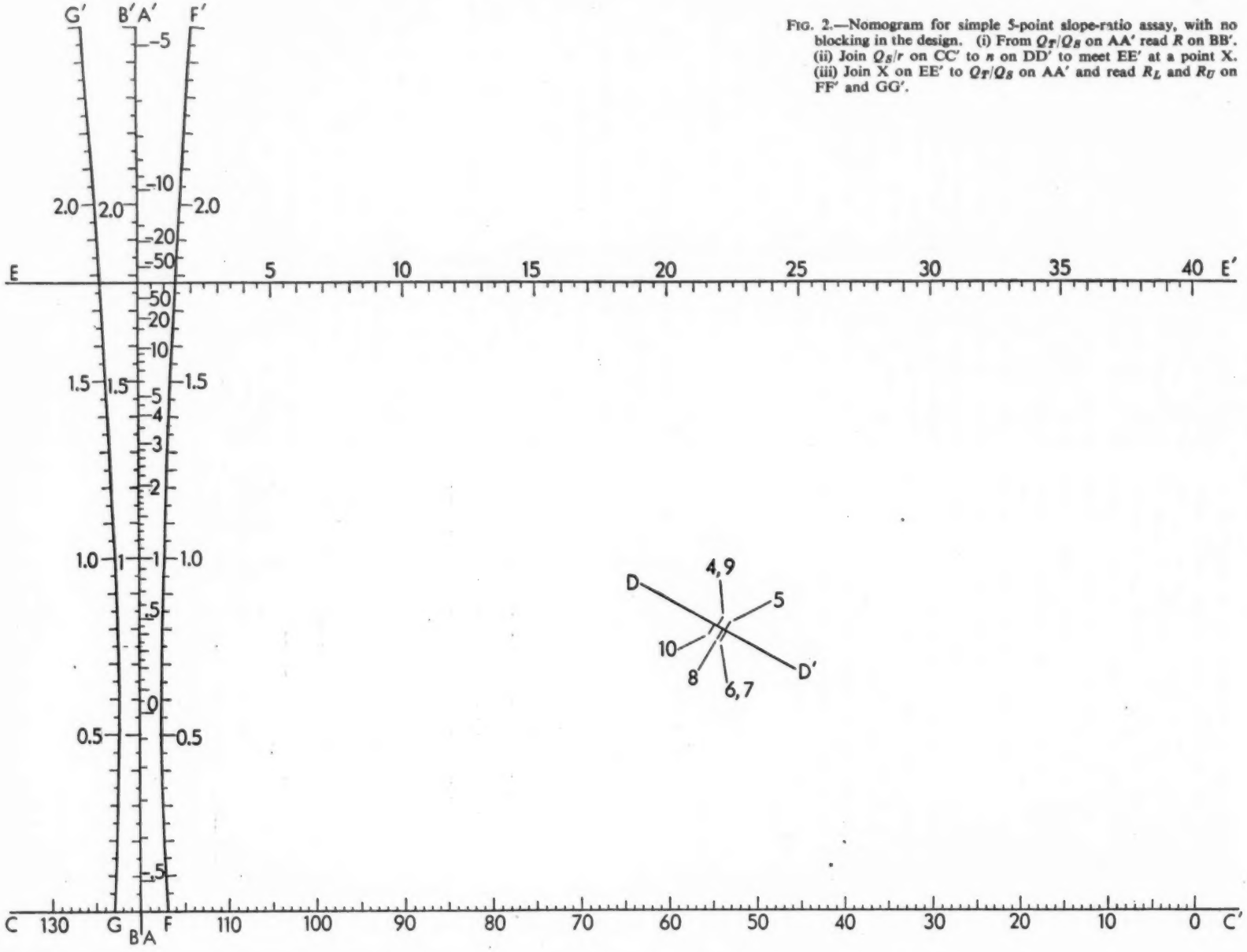

FIG. 2.—Nomogram for simple 5-point slope-ratio assay, with no blocking in the design. (i) From Q_T/Q_S on AA′ read R on BB′. (ii) Join Q_S/r on CC′ to n on DD′ to meet EE′ at a point X. (iii) Join X on EE′ to Q_T/Q_S on AA′ and read R_L and R_U on FF′ and GG′.

to suit other dimensions is made by multiplication of all vertical or horizontal scales by some suitable conversion factor.

Multiple Slope-ratio Assay, arranged in Blocks

The type of design to be discussed is illustrated in the first numerical example. There is a common zero dose, and each preparation is represented at two dose-levels; the doses are equally spaced on an arithmetic scale, so that they may be regarded as 0, $\frac{1}{2}$, and 1 units. Using the notation of earlier papers, let v denote the number of preparations, including the standard: the tables cover values of v from 3 to 16 inclusive. The assay is arranged in two blocks, and in each block there is one observation at each level of each test preparation and $(p+1)$ observations at the zero dose and at each level of the standard preparation, where $(p+1)$ is taken approximately equal to $\sqrt{(v-1)}$, as recommended for multiple assays (Finney, 1952).

The method of calculation of the quantity r required for using the nomogram has been described in the example. The only other basic quantities required are values of Q for each preparation, from which the total $S(Q)$ and ratios $S(Q)/Q$ are calculated. These values of Q are given by the equations

$Q_S=(3p+2v+1)(S_1+2S_2)-3(p+1)G$, for the standard and $Q_{Ti}=(3p+2v+1)(T_{i1}+2T_{i2})-3G$, for the ith test preparation ($i=1, 2, \ldots, v-1$),

TABLE IV

RELATIVE POTENCY SCALE FOR MULTIPLE SLOPE-RATIO ASSAY NOMOGRAMS

R	y	R	y	R	y	R	y
0·30	20·00	0·50	12·00	0·70	8·57	1·25	4·80
0·31	19·35	0·51	11·76	0·72	8·33	1·30	4·62
0·32	18·75	0·52	11·54	0·74	8·11	1·35	4·44
0·33	18·18	0·53	11·32	0·76	7·89	1·40	4·29
0·34	17·65	0·54	11·11	0·78	7·69	1·45	4·14
0·35	17·14	0·55	10·91	0·80	7·50	1·50	4·00
0·36	16·67	0·56	10·71	0·82	7·32	1·60	3·75
0·37	16·22	0·57	10·53	0·84	7·14	1·70	3·53
0·38	15·79	0·58	10·34	0·86	6·98	1·80	3·33
0·39	15·38	0·59	10·17	0·88	6·82	1·90	3·16
0·40	15·00	0·60	10·00	0·90	6·67	2·00	3·00
0·41	14·63	0·61	9·84	0·92	6·52	2·20	2·73
0·42	14·29	0·62	9·68	0·94	6·38	2·40	2·50
0·43	13·95	0·63	9·52	0·96	6·25	2·60	2·31
0·44	13·64	0·64	9·38	0·98	6·12	2·80	2·14
0·45	13·33	0·65	9·23	1·00	6·00	3·00	2·00
0·46	13·04	0·66	9·09	1·05	5·71	3·50	1·71
0·47	12·77	0·67	8·96	1·10	5·45	4·00	1·50
0·48	12·50	0·68	8·82	1·15	5·22	4·50	1·33
0·49	12·24	0·69	8·70	1·20	5·00	5·00	1·20
						10·00	0·60

where S_1, S_2 are the totals for the lower and upper doses respectively of the standard preparation, T_{i1} and T_{i2} are the corresponding totals for the ith test preparation and G is the total of all observations.

Rectangular coordinate axes are drawn through an origin on the base-line of the chart and 6 units from the left-hand edge. All subsequent lines and scale marks, summarized in Tables III and IV, will be described by reference to these axes.

Two scales are marked along the x axis. The first, along the lower side, is a linear scale for b_S/r,

TABLE III

COORDINATES AND SCALE FACTORS FOR MULTIPLE SLOPE-RATIO ASSAY NOMOGRAMS

v (No. of preparations including standard)	3	4	5	6	7	8	9	10	11	12	13	14	15	16
$p+1$ (No. of replications, per block, of standard and blank)	1	2	2	2	2	3	3	3	3	3	3	4	4	4
Scale for b_S/r: linear scale along x-axis from $x=0$														
Scale factor	2	3	3	4	4	4	4	4	4	5	5	5	5	5
Scale for $S(Q)/Q_S$: linear scale along x-axis from $x=X$														
X	4·44	5·00	4·44	3·78	4·00	4·63	3·85	4·00	4·15	3·22	3·33	4·11	4·22	3·25
Scale factor	5	6	5	4	4	5	4	4	4	3	3	4	4	3
Scale for R: inverse scale along y-axis; scale marks given in Table IV.														
Scale for b_S: linear scale along y-axis; scale factor θ chosen to suit data.														
Scale for $S(Q)$: linear scale along vertical line $x=X$ (see values of X above).														
Scale factor	$\theta\times$0·03214	0·01000	0·00804	0·0062	0·00556	0·00277	0·00247	0·00222	0·00200	0·00182	0·00166	0·00128	0·00118	0·00110
Scale for $S(Q)/Q_T$: non-linear scale along vertical line $x=X$. Scale given by $y=\{6S(Q)/Q_T\}/\{\psi+S(Q)/Q_T\}$														
ψ	0·$\dot{8}$	1·$\dot{6}$	1·$\dot{7}$	1·$\dot{8}$	2·0	2·$\dot{7}$	2·$\dot{8}$	3·0	3·$\dot{1}$	3·$\dot{2}$	3·$\dot{3}$	4·$\dot{1}$	4·$\dot{2}$	4·$\dot{3}$
Curves for R_L and R_U: symmetrical curves about y-axis. Scale marks as for R.														
Coordinates of points on curves														
$y=0$, $\pm x=$	1·32	0·96	0·74	0·83	0·73	0·57	0·52	0·49	0·47	0·55	0·53	0·46	0·44	0·43
$y=1·2$, $\pm x=$	1·20	0·88	0·68	0·77	0·68	0·53	0·49	0·46	0·44	0·52	0·50	0·44	0·42	0·41
$y=3·0$, $\pm x=$	1·$\dot{5}$2	0·87	0·68	0·77	0·68	0·56	0·52	0·49	0·47	0·56	0·54	0·49	0·48	0·46
$y=6·0$, $\pm x=$	1·28	1·13	0·88	1·00	0·88	0·79	0·73	0·70	0·66	0·80	0·77	0·73	0·71	0·69
$y=10·0$, $\pm x=$	1·87	1·73	1·34	1·54	1·37	1·23	1·15	1·09	1·04	1·25	1·21	1·15	1·12	1·10
$y=15·0$, $\pm x=$	2·82	2·62	2·02	2·30	2·05	1·85	1·72	1·63	1·56	1·87	1·80	1·73	1·68	1·64
$y=20·0$, $\pm x=$	3·86	3·54	2·74	3·11	2·77	2·49	2·32	2·20	2·09	2·51	2·42	2·32	2·26	2·20
Coordinates of the turning-point														
y	3·18	2·25	2·16	2·08	2·00	1·59	1·54	1·50	1·46	1·42	1·38	1·17	1·15	1·12
$\pm x$	1·12	0·86	0·66	0·75	0·67	0·53	0·49	0·46	0·44	0·52	0·50	0·44	0·42	0·41
Validity tests (i) $\lvert S(H)-AT_\theta\rvert \leqslant t_1 r$; (ii) $\lvert S(H)-AH_S\rvert \leqslant t_2 r$; (iii) Range $(H_T)\leqslant t_3 r$														
A	3·0	2·5	3·0	3·5	4·0	3·$\dot{3}$	3·$\dot{6}$	4·0	4·$\dot{3}$	4·$\dot{6}$	5·0	4·25	4·50	4·75
t_1	4·94	4·20	3·69	3·53	3·47	3·47	3·47	3·50	3·55	3·60	3·67	3·65	3·72	3·78
t_2	5·53	4·20	4·13	4·28	4·47	4·11	4·30	4·52	4·74	4·95	5·20	4·84	5·04	5·24
t_3	5·00	3·09	2·54	2·33	2·14	2·03	1·95	1·88	1·83	1·79	1·75	1·72	1·70	1·68

with scale factor as shown in Table III. Thus, for 6 test preparations and $p=1$, the scale factor is 4, so that the marks for $b_S/r=1, 2, 3, \ldots$ fall at $x=4$, 8, 12, .. respectively, and convenient intermediate scale marks are easily located. Along the upper side of the x-axis is a linear scale for $S(Q)/Q_S$, which starts, not at the origin, but at $x=X$, where X and the appropriate scale factor are given in Table III. Taking the same example, we have $X=4.00$ and the scale factor is 4, so that the marks for $S(Q)/Q_S=0$, 0.5, 1.0, 1.5, .. fall at $x=4.00$, 6.00, 8.00, 10.00, .. respectively.

There are also two scales along the y-axis. On the right-hand side is an inverse scale for R, the relative potency estimate, and the positions of the marks for this scale, which are unaltered for different values of v and p, are given in the separate Table IV. A linear scale for b_S, the slope of the dose-response line for the standard preparation, is marked along the left-hand side of the y-axis, and here the scale factor is chosen to suit the data. The scale factor θ may conveniently be chosen as a round number such that, if Δ is the maximum difference expected between the mean for the highest dose of the standard and the mean for the zero dose in any one assay, then θ is approximately equal to $20/\Delta$. The choice of the value of θ clearly depends on the units of observation. Thus if the maximum mean difference expected was about 100, the value of θ would be taken to be 0.2, as in the numerical example in this paper.

A vertical line is drawn through the point $x=X$, $y=0$ previously located as the starting point of the scale for $S(Q)/Q_S$. A linear scale for $S(Q)$ is marked on its left-hand side; the scale factor depends on the value chosen for θ, and the factors listed in Table III have to be multiplied by θ. Thus if $v=7$ and $p=1$, and if θ is taken as 0.2, the scale factor for $S(Q)$ is 0.2×0.00556, i.e. 0.00112, so that the marks for $S(Q)=0$, 1,000, 2,000, .. fall at $y=0$, 1.12, 2.24, .. respectively. Space does not permit the listing of the coordinates of the scale points for $S(Q)/Q_T$, which are marked on the right-hand side of this vertical line; they may be easily computed from the formula $y=\dfrac{6S(Q)/Q_T}{\psi+S(Q)/Q_T}$ where the value of ψ for any particular case is given in Table III. Taking the same example, ψ is seen to be equal to 2, so that the scale marks for $S(Q)/Q_T=0$, 1, 10, ∞, -10 fall at $y=0$, 2.00, 5.00, 6.00, 7.50 respectively.

It remains only to draw the curves for R_L and R_U, the lower and upper fiducial limits, here determined, as usual, for the 5% probability level. These curves are symmetrical about the y-axis, and since they are smooth they may be adequately defined by only a few coordinates. The values of x for seven particular values of y are listed in Table III, and in addition the coordinates are given of the point, for each curve, at which the horizontal distance from the y-axis is a minimum (the turning-point). From these values it should be possible to draw the curves with sufficient accuracy, and marking them with scales is an easy matter, since the scales for R_L and R_U are the same, in the vertical direction, as the scale for R.

The nomogram is then complete, and the method of use is illustrated in the numerical example and summarized in the legend to Fig. 1.

Simple Slope-ratio Assay, not arranged in Blocks

When there is only one test preparation, the resulting simplification of the general method makes it possible to draw up a single nomogram for use with any number of replications. As mentioned earlier, the cases to be considered here are of 5-point assays not arranged in blocks, the usual design for simple microbiological assays.

The quantities Q required for entering the nomogram are calculated as for multiple assays, taking $p=0$ and $v=2$, so that for the standard preparation, for example, $Q_S=5(S_1+2S_2)-3G$. The method of calculating r is changed, since there are no blocks: r is given by the sum of the 5 ranges of n observations within each treatment, where n is the number of replications.

Assuming, again, a sheet approximately 20 by 30 units in size, the origin is taken on the base line, 4 units from the left-hand edge. Rectangular axes are drawn through the origin, and a horizontal line is also drawn at $y=14.22$.

The y-axis bears, on the left-hand side, a linear scale for R, with scale factor 8, and on the right-hand side, a scale for Q_T/Q_S given by $y=\dfrac{8(9+16Q_T/Q_S)}{16+9Q_T/Q_S}$. A number of scale marks for Q_T/Q_S have been tabulated in Table V.

Coordinates sufficient to draw the curves for R_L and R_U, which are symmetrical about the y-axis, are also given in Table V, and include the coordinates of the turning-point. The curves so defined refer to the 5% probability level. The scale marks are simply located by the same vertical scale as that for R.

Any convenient linear scale is marked off for Q_S/r on the x-axis, running in the negative direction as shown in Fig. 2, and starting at $x=24$. The upper horizontal line is graduated with a linear scale for an intermediate variable, β, starting at $x=0$, and with a scale factor 0.6.

TABLE V

COORDINATES AND SCALE FACTORS FOR SIMPLE SLOPE-RATIO ASSAY NOMOGRAMS

Scale for R: linear scale along y-axis, with scale factor 8.
Scale for Q_T/Q_S: non-linear scale along y-axis. Scale marks given below.

Q_T/Q_S	y	Q_T/Q_S	y	Q_T/Q_S	y	Q_T/Q_S	y
−0·5	0·70	0·50	6·63	2·0	9·65	15	13·19
−0·4	1·68	0·6	6·95	2·2	9·88	20	13·43
−0·3	2·53	0·7	7·25	2·4	10·09	50	13·89
−0·2	3·27	0·8	7·52	2·6	10·27	∞	14·22
−0·1	3·92	0·9	7·77	2·8	10·45	−50	14·58
0·0	4·50	1·0	8·00	3·0	10·60	−20	15·17
0·05	4·77	1·1	8·22	3·5	10·95	−15	15·53
0·10	5·02	1·2	8·42	4·0	11·23	−10	16·32
0·15	5·26	1·3	8·61	4·5	11·47	−9	16·62
0·20	5·51	1·4	8·78	5	11·67	−8	17·00
0·25	5·70	1·5	8·95	6	12·00	−7	17·53
0·30	5·90	1·6	9·11	7	12·25	−6	18·32
0·35	6·10	1·7	9·25	8	12·45	−5	19·59
0·40	6·29	1·8	9·39	9	12·62		
0·45	6·46	1·9	9·52	10	12·75		

Curves for R_L and R_U: symmetrical curves about y-axis. Scale marks as for R.
Coordinates of points on curves:

y	4·50 Turning-	0·00	2·00	6·00	8·00	12·00	16·00	20·00
x	0·48 point	0·60	0·53	0·51	0·56	0·75	0·99	1·26

Scale for Q_S/r: linear scale along x-axis in negative direction, starting at $x=24$.
Scale factor φ chosen for convenience.
Scale for β: linear scale along horizontal line $y=14{\cdot}22$, with scale factor 0·6.
Scale for n: intersections of line joining $Q_S/r=0$ and $\beta=0$ with line joining points $x=10$, $y=0$ and $x=\lambda/\varphi$, $y=14{\cdot}22$. Values of λ given below.

n:	4	5	6	7	8	9	10
λ:	3·367	3·467	3·485	3·475	3·442	3·383	3·340

Validity tests: (i) $|S(H)-2T_0| \leqslant t_1 r$; (ii) $|H_S-H_T| \leqslant t_2 r$

t_1:	1·53	1·49	1·48	1·49	1·50	1·53	1·55
t_2:	1·30	1·26	1·25	1·26	1·27	1·29	1·31

The nomogram is completed by marking off a scale for n, the number of replications, on a straight line joining the points corresponding to $Q_S/r=0$ and $\beta=0$. The scale marks are made at the intersections of this line with a straight line joining the point $x=10$ on the x-axis to the point on the upper horizontal line with x-coordinate equal to λ/φ, where φ is the scale factor chosen for Q_S/r and λ is given in Table V.

This nomogram is applicable to any value of n. If it is only required to make a chart suitable for one particular value of n, the scale on the x-axis may be omitted, and a linear scale for Q_S/r substituted for the β-scale on the horizontal line at $y=14{\cdot}22$. The scale factor is then taken as $\lambda/14$ where λ is given in Table V.

The method of use of the nomogram is explained in the legend to Fig. 2 and is illustrated in the second example.

VALIDITY TESTS

The validity tests are made in much the same way as described in the earlier paper (Clarke and Hosking, 1953), except that it is necessary to replace Student's t by Lord's u (Lord, 1947) in the tests since ranges have been used to estimate the error variance. Unequal replication and arrangement in blocks, in the case of the multiple assay, introduce a few complications, but the application of the tests has been simplified here by listing the critical values which arise in the tests. These values have been calculated for the 5% level of probability. For an explanation of the tests for " blanks " and " intersections " the reader is referred to Finney (1952).

Multiple Slope-ratio Assays

As a preliminary step, the quantity H is calculated from the treatment totals for each preparation, where, for example,

$$H_S=2S_1-S_2.$$

The test for " blanks " requires that $S(H)-AT_0$ should not be numerically greater than $t_1 r$, where A and t_1 are given in Table III, $S(H)$ is the sum, over preparations, of the values of H, and T_0 is the sum of the observations for the zero dose.

The test for intersections may be divided into two parts, first comparing the standard preparation with the mean of the test preparations and next making comparisons among the test preparations. Thus $S(H)-AH_S$ should not be numerically greater than $t_2 r$, and the range of the values of H for the test preparations only should not be greater than $t_3 r$: the values of t_2 and t_3 are given in Table III.

Simple Slope-ratio Assays

The quantity H for each preparation is calculated in the same way as for multiple assays. The tests for " blanks " and " intersections " then require, respectively, that $S(H)-2T_0$ should not be numerically greater than $t_1 r$ and that H_S-H_T should not be numerically greater than $t_2 r$ where t_1 and t_2 are given in Table V.

OTHER DESIGNS

The general forms of the nomograms for slope-ratio assays are unaltered by differences in design. The range function used to estimate the error variance depends on whether or not the assay is arranged in blocks, and this correspondingly affects the scale for Q_S/r (multiple assays) or n (simple assays). In a simple assay, the error variance may be easily estimated from ranges for any number of blocks, and a multiple assay without blocks may also be simply dealt with regardless of the number of replications. Another type of design to which range methods are easily applicable is that of multiple assays arranged in more than two blocks and with more than two non-zero dose-levels; for only two dose-levels and more than two blocks, however, range methods become cumbersome, and it may often be simpler to calculate the error mean square from an analysis of variance, although a nomogram will still be found helpful for estimating fiducial

limits, particularly if there is a large number of preparations.

Using similar methods as for multiple slope-ratio assays, nomograms may be developed for multiple parallel-line assays, in which the response is linearly related to the logarithm of the dose. The basic formulae may be obtained as described by, for example, Finney (1952), and used to adapt the nomogram presented by Leech and Grundy (1953). When the assay is arranged in blocks, the estimation of the error variance from ranges is complicated if the number of dose-levels per preparation exceeds 2, unless only the deviations from linearity of the dose-response curves are used to estimate the variance. With multiple assays, it is not in general possible to make one nomogram to serve for several values of n, as with the simple assay, and so the scale for γ (Leech and Grundy, 1953) is replaced by a scale for a function analogous to R/B in that paper.

Summary

Graphical methods for the evaluation of results of multiple slope-ratio assays arranged in randomized blocks are presented, and the development of similar methods for simple slope-ratio assays arranged in blocks and for multiple parallel-line assays is discussed. Improved nomograms for simple assays without blocks are also described, and the modifications for multiple assays without blocks are outlined. The methods for multiple assays allow for increased replication of the standard preparation, and validity tests are given for use in all cases considered. Numerical examples illustrate the procedures.

I wish to thank Mr. C. P. Cox for helpful discussions, Dr. M. E. Gregory for the data used in the examples, Miss J. King for computing assistance and Miss P. J. Edwards and Mr. G. W. Coote for drawing the diagrams.

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THE EFFECTS OF GLYCYRRHETINIC ACID ON SALT AND WATER METABOLISM

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(RECEIVED MARCH 10, 1955)

Revers (1948) observed that succus liquiritiae caused water retention in some peptic ulcer patients. Molhuysen, Gerbrandy, de Vries, de Jong, Lenstra, Turner, and Borst (1950) confirmed this and found an extract consisting mainly of glycyrrhizin to be equally effective.

In normal persons, the authors reported that the succus made the potassium balance markedly negative and the sodium balance strongly positive. They failed to produce similar results in a patient with Addison's disease and concluded that the drug acted through the adrenals.

Groen, Willebrands, and Kamminga (1951), and Groen, Frenkel, Kamminga, and Willebrands (1952) found liquorice to have a DOCA-like effect on sodium and potassium metabolism in normal people and in Addisonian patients. They found glycyrrhizinic acid to be the active principle and used it for the treatment of Addison's disease.

Borst, ten Holt, de Vries, and Molhuysen (1953) suggested that liquorice activity on salt and water metabolism is potentiated by the endogenous corticoids. No conclusive evidence of such a potentiation has been found in the treatment of rheumatoid arthritis (Hart and Leonard, 1954) or in the increase in the urinary output of 17-ketosteroids (Hudson, Mittelman, and Mann, 1953).

Calvert (1954) successfully maintained a patient with Addison's disease on liquorice extract for over a year. The daily maintenance dose decreased from 60 g. to 3 g. The author concluded that the drug has a place in long-term therapy of this disease.

Glycyrrhizinic acid is a conjugate of glycuronic acid and glycyrrhetinic acid ; the latter was found to have a DOCA-like effect in normal people and in Addisonian patients (Card, Mitchell, Strong, Taylor, Tompsett, and Wilson, 1953).

Nelemans and Stamperius (1949) and Card *et al.* (1953), however, found no evidence of any effect of liquorice or glycyrrhetinic acid on water and electrolyte metabolism in laboratory animals.

Glycyrrhetinic acid is not a steroid and therefore it would be of great interest if it could be shown to possess some of the more important properties of the corticosteroids.

METHODS

Urinary Sodium Excretion

The details of the method used for assaying mineralo-cortical activity will be reported separately. Young female albino rats, adrenalectomized under ether anaesthesia three days before the assay, were used. The rats were divided into groups of 5–7 animals each. On the day of the experiment, each rat was given two loads of the electrolytes investigated. Each group received either a known quantity of glycyrrhetinic acid dissolved in propylene glycol or a known quantity of DOCA in the same volume of the solvent. A comparable group of animals was included in every experiment and received the same quantity of solvent alone.

The urine of each animal was collected separately over a period of six hours and its sodium or potassium content was estimated by the flame-photometer. In some experiments, the urine was collected for 4 hr. only.

Blood Electrolytes

Male albino rats weighing 300 g. were adrenalectomized under ether anaesthesia and kept on rat cubes and a drinking solution containing sodium chloride (1%) and glucose (5%) for 4 days. On the 5th day, the animals were weighed and divided into three groups of equal mean weight. Injections were started and continued daily for five days. No saline drink was administered during this period.

Group 1 rats received subcutaneous injections of 0.25 ml. propylene glycol. Group II received 3 mg. glycyrrhetinic acid dissolved in 0.25 ml. propylene glycol. Group III received 1 mg. of DOCA in 0.25 ml. propylene glycol. Group IV rats were non-adrenalectomized, from the same colony, and of the same weight. Each rat received 0.25 ml. of propylene glycol subcutaneously.

On the 5th day, 4 hr. after the injection, the animals were anaesthetized with an intraperitoneal injection of 0.7 ml. 6% pentobarbitone sodium solution. The chest was opened and the heart punctured. The blood was collected in a centrifuge tube and allowed to clot ;

* In receipt of a scholarship from Cairo University.

the serum was pipetted off later. After dilution, the sodium and potassium contents were estimated with the flame photometer.

Diuresis

(*a*) *Anaesthetized Rats.*—Male rats above 250 g. were used. They were fasted overnight, but given free access to tap water. At about 10 a.m. anaesthesia was produced by administering 5 ml./100 g. body weight of 10% alcohol in tap water into the stomach by a thin rubber catheter, and the rat was kept in a warm place. If the rat was not unconscious in 45 min., another dose was given (1.5 ml. of the same solution/100 g. body weight). A small suprapubic cut was made, the bladder secured and a thin polythene tube (3 mm. internal diameter) was introduced into the bladder through the apex and tied in securely. The penis was tied to prevent any urine escaping except through the polythene tube. A thin polythene cannula was put into the jugular vein. A fine thermometer was introduced into the rectum and the animals were placed on a box warmed by electric bulbs and their temperature kept as constant as possible at 37°. The end of the catheter was fixed to a graduated centrifuge tube and a reading of the urine collected was taken every 10 min. When diuresis started, a new water load of 10–14 ml. of water, or of 3% alcohol in tap water if anaesthesia was getting light, was introduced by stomach tube. The degree of anaesthesia was kept deep enough to prevent the animals from moving or responding to external stimuli, but not so deep as to inhibit diuresis. Whenever 8 ml. of urine had been collected, it was replaced by 10 ml. of water by stomach tube or of 3% alcohol, if required. The solvent of the drug was administered intravenously in the same manner as the drug, which was given as a clear solution in propylene glycol (0.05–0.2 ml.) and washed down the cannula with a minimum amount of saline. Care was taken, as all these factors affected the flow of urine.

(*b*) *Conscious Rats.*—Female albino rats around 150 g. weight were used. Food was withdrawn at 5 p.m. the previous night; water was withdrawn at 9 a.m. on the day of the experiment. The animals were divided into two equal groups, one to act as control and the other as test. At 11 a.m. each control rat was given 1 ml. of propylene glycol by stomach tube. The test group had 100 mg. glycyrrhetinic acid dissolved in 1 ml. of propylene glycol. The rats were kept in a quiet place for 3 hr. At 2 p.m. each animal got 12 ml. of tap water by stomach tube; its bladder was emptied by a gentle pulling of the tail, and the rat was placed in a metabolism cage which consisted of a waxed tin funnel covered by a waxed wire mesh and surmounted by a cylinder of wire mesh with a tin cover. A graduated cylinder was placed under each cage to receive the urine. Two hours after placing the animals in the cages the bladder was emptied by pulling the tail gently, and the urine was allowed to run into the cylinder; then a reading was taken. The whole experiment was carried out at constant temperature (22° C.±1°) in a quiet room. Five days later, after 4 days of complete rest, the experiment was repeated and the groups were crossed so that each animal acted as its own control. To eliminate any source of emotional stress that may interfere with the diuresis, it was necessary first to train the animals to the conditions of the experiment, particularly the use of the stomach tube, for a week.

(*c*) *Normal Dog.*—A collie bitch of 10 kg. weight was used. A period of 3 weeks' training was necessary before the full co-operation of the dog was gained.

The night before the experiment, food was removed from the cage; the dog had free access to water. At 9 a.m. the next morning, 70 ml. of tap water, divided into three portions, was given by stomach tube. Glycyrrhetinic acid (350 mg.) was suspended in the first portion and washed down by the last two. The dog was then allowed free movement with no access to food or water for 2 hr. At 11 a.m. a self-retaining catheter was introduced and the bladder emptied. The dog was placed in a Pavlov stand. At 11.15 a.m. 250 ml. of tap water was given by stomach tube, followed by 300 ml. at 11.55. From 12 noon, urine flow was recorded at 5 min. intervals for 1 hr. and 40 min. The total urine excreted from 11.15 till 3.05 was also measured. Throughout the experiment, everything was avoided which might antagonize the dog and all external interferences were cut down as far as possible. A constant temperature and complete quietness were essential.

In one experiment in which the dog received no glycyrrhetinic acid, 2.5 mg. DOCA was injected subcutaneously at 9 a.m. and the rest of the experiment continued as before.

(*d*) *Rats with Diabetes Insipidus.*—Female albino rats (120–150 g.) were used. The posterior lobe of the pituitary gland was sucked out under ether anaesthesia, most of the anterior lobe being left intact. The operation was kindly performed by Dr. M. Vogt. The rats were given 5% glucose drinking solution for 2 days and ordinary rat cubes for food. Penicillin was administered as a protective measure for 2 days. In order to discover which rats were diabetic, 2 days after the operation each rat was put in a metabolism cage and was freely supplied with water and cubes. Urine was collected over 24 hr. and the volume compared with that from intact rats of the same batch. Later, collection of urine was carried out over-night for 18 hr. with water only supplied, the feeding being done late every afternoon. The urine volume was again compared with that of intact control rats. Overnight urine collections were continued during the glycyrrhetinic acid tests to measure the degree of diabetes insipidus present in every test rat during the whole procedure. This was necessary because in some of the operated rats only a transient phase of diabetes was achieved.

The test for the antidiuretic action of glycyrrhetinic acid was carried out only on those rats in which the operation proved successful. All the rats used in these tests were diabetic throughout the test period.

The method used to test the effect of glycyrrhetinic acid on diuresis in the rats with diabetes insipidus was the same as that employed for the non-anaesthetized intact rats. The same dose (100 mg.) of the acid was introduced by stomach tube 3 hr. before the 12 ml. water load. A 2 hr. urine collection was carried out. Each rat acted alternately as control and as test animal. Two control values and two test values were obtained from each rat whenever possible. Before subjecting the rats to the operation they were trained for one week to get them accustomed to the stomach tube and urine collection procedures.

Water Absorption from the Alimentary Canal

Normal Rats.—Adult albino rats of about 170 g. weight were trained for one week to being given water by stomach tube. The actual experiment consisted in fasting the rats overnight, allowing them free access to drinking water. They were then divided into pairs, selected to be identical in sex and weight. At 11 a.m. 1 ml. of propylene glycol was administered by stomach tube to one rat of each pair and 100 mg. of the acid suspended in the same volume of propylene glycol to the other. The rats were kept in a quiet, warm place for 3 hr., when they received 5% of the body weight of warm tap water by stomach tube. They were killed 15 to 60 min. later and their intestinal tracts were dissected out and weighed after removing the formed contents of the rectum and caecum.

Throughout the experiment, the two rats of each pair were handled together and kept in the same cage; conditions for the members of the same pair were therefore as identical as possible and the results obtained for the control and the glycyrrhetinic-treated rats comparable.

The dose of the acid, its mode of administration, and the period elapsing until the administration of the water load were chosen so as to duplicate the procedures in the diuresis experiments reported before, in the hope that such an arrangement would facilitate the assessment of the results.

In another series of experiments, a 300-mg. dose of the acid was given to the test animals, the contents of the caecum were not emptied before weighing the gut, and the liver was dissected out and weighed. The times allowed for water absorption were 15, 30, and 75 min.

RESULTS

Urinary Sodium Excretion

In glycyrrhetinic-acid-treated adrenalectomized rats (Table I) sodium excretion was not inhibited or altered to any significant degree if the collection of urine was continued for a period sufficiently long to allow urine to flow. With doses of 0.2 and 0.4 mg. glycyrrhetinic acid a 6 hr. collection was sufficient. When DOCA, 1.0 μg./rat, was administered under the same conditions significant sodium retention was achieved ($P=0.03$).

TABLE I

SODIUM EXCRETION IN ADRENALECTOMIZED RATS

Expt. No.	Time of Collecting Urine (hr.)	Propylene Glycol Only		Glycyrrhetinic Acid (GA) or DOCA			
		No. of Rats	Mean Na Excretion (mg.)	No. of Rats	Dose (μg.)	Mean Na Excretion (mg.)	Na Excretion (% of Control)
1	6	7	3·45	8	400 GA	3·78	109·6%
2	6	6	0·61	6	200 GA	1·45	240·0%
3	6	7	2·66	7	200 GA	2·25	84·6%
				7	1·0 DOCA	0·899	29·6%
4	6	5	2·53	6	400 GA	2·23	89·2%
				5	5·0 DOCA	0·573	22·6%
5	6	6	1·58	6	400 GA	1·29	84·3%
6	4	7	1·48	4 and 3*	400 GA	0·56	37·8%
7	4	7	1·66	4 and 3*	400 GA	0·39	23·5%
				7	1·5 DOCA	0·43	26·0%

* Number of animals which excreted no urine at all. They are included in the calculation of the mean Na excretion for the whole group.

When urine was collected over 4 hr. only, and a dose of 0.4 mg. of the acid was administered to each rat, the mean sodium excretion for the whole group was appreciably below that of the control group. Under these conditions, however, it was observed that a number of the glycyrrhetinic-acid-treated rats did not excrete any urine at all and thus did not contribute to the total excretion of sodium by the group. With a 4 hr. collection, 1.5 μg. DOCA caused a significant sodium retention ($P<0.01$), while the volume of urine collected from any rat was not appreciably smaller than that collected in 6 hr. In other experiments, the second sodium load was replaced by a load of 3.8 mg. potassium chloride in 2.0 ml. water. The group of rats receiving 400 μg. of glycyrrhetinic acid showed a significant increase in their mean urinary potassium output compared with the control group ($P<0.02$). See Table II.

TABLE II

POTASSIUM EXCRETION IN ADRENALECTOMIZED RATS AFTER A KCl LOAD OF 3·8 MG.

Controls				Glycyrrhetinic Acid (400 μg.)			
No. of Rats	Mean Na Excretion (mg.)	Mean K Excretion (mg.)	$\frac{K}{Na} \times 100$	No. of Rats	Mean Na Excretion (mg.)	Mean K Excretion (mg.)	$\frac{K}{Na} \times 100$
6	1·05	0·86	81·9	6	1·62	2·41	148·8

Blood Electrolytes

The results are shown in Table III. DOCA in 1.0 mg. doses given for five days successfully maintained the serum sodium of adrenalectomized rats above the normal level for the non-adrenalectomized rats of the same colony ($P<0.01$). The

TABLE III

EFECTS OF GLYCYRRHETINIC ACID (GA) ON SERUM ELECTROLYTES IN RATS

	Group	No. of Rats/ Group	Mean Serum Na (% of Normal)	Significance of Na Change	Mean Serum K (% of Normal)	Significance of K Change	Mean Gain in Weight (g.)	Survival %
Normal	0·25 ml. propylene glycol	8	100	—	100	—	+0·1	100
Adrenalectomized	0·25 ml. propylene glycol	10	88·7	From normal $P<0·001$	166·7	From normal $P=0·01-0·02$	−10·5	30
	1 mg. DOCA	8	105·9	From adrenalectomized non-treated $P<0·001$ From normal $P<0·01$	144·7	From adrenalectomized, non-treated—not significant From normal—not significant ($P=0·1-0·2$)	+0·5	100
	3 mg. GA	10	91·3	From adrenalectomized non-treated—not significant From normal $P<0·01$	163·5	From adrenalectomized non-treated—not significant From normal $P=0·2$	−8·6	50

The doses are per day per rat of approx. 300 g. All injections were given subcutaneously for 5 days.

serum sodium of the DOCA-treated adrenalectomized rats was thus very much higher than that of the adrenalectomized propylene-glycol-injected controls ($P<0.001$). These controls showed the classical picture of adrenal deficiency; their serum sodium was below the normal level ($P<0.001$) and their serum potassium higher ($P<0.001$). The serum potassium of the DOCA-treated group showed a slight return towards normal, but was not significantly different from that of untreated adrenalectomized or normal rats.

Glycyrrhetinic acid did not cause a significant rise of serum sodium or a fall of serum potassium. The daily dose administered was equivalent to 600 mg. glycyrrhetinic acid for a 60 kg. man, a dose well within the range reported to be effective in man.

DOCA successfully maintained all the animals in the group alive and in good shape. The glycyrrhetinic acid group started to show weakness much earlier even than the adrenalectomized controls. The survival of the group, however, was slightly, but not significantly, longer than that of the controls.

The adrenalectomized animals treated with glycyrrhetinic acid or left without treatment showed a marked loss of weight; this was prevented by DOCA.

Diuresis

(*a*) *Anaesthetized Rats.*—Glycyrrhetinic acid caused an appreciable inhibition of diuresis. In a dose of 1 mg. intravenously to a 300 g. rat, it caused almost complete suppression of urine flow from which recovery was very slow, taking about 3–4 hr. The response, however, was not uniform. Some rats responded to a small dose of 200 μg. more markedly than other rats to bigger doses.

When given intravenously (Fig. 1) 0.1 ml. propylene glycol alone had no effect, whereas, when the same fluid contained 100 μg. of the acid, the diuresis diminished appreciably for 4 hr. and then did not regain the initial level, although this had previously been maintained practically constant for 2 hr.

The smallest effective dose for a 280 g. rat was around 40 μg. (Fig. 2); 150 μg. had a marked effect on this rat.

When the drug was injected intravenously, a slight haemorrhagic tinge was sometimes noticed in the urine. This tinge, however, was also noticed with propylene glycol alone.

(*b*) *Conscious Rats.*—The results are shown in Table IV. A variable degree of water retention

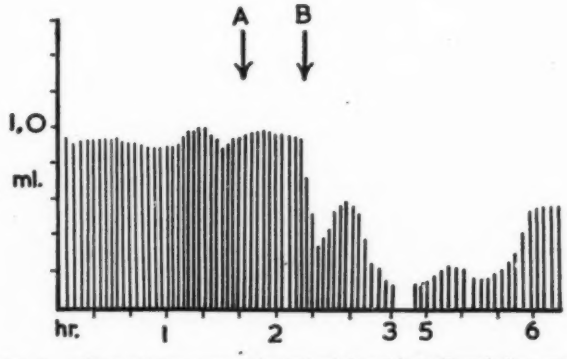

FIG. 1.—Effect of intravenous glycyrrhetinic acid on diuresis in a rat. Ordinate: Urine volume in ml./10 min. Abscissa: Time in hr. A: 0·1 ml. propylene glycol washed in with 0·6 ml. saline. B: same as A, but containing 100 μg. glycyrrhetinic acid.

TABLE IV

EFFECT OF GLYCYRRHETINIC ACID (GA) BY MOUTH ON DIURESIS IN NORMAL UNANAESTHETIZED RATS

Urine collected over 2 hr. after a load of 12 ml. H_2O.

No. of rat	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Mean
Controls (ml. urine)	8	10	10	7·5	8	6·5	8·5	9	7·5	8·5	9·5	6	9	8	8·29
100 mg. GA (ml. urine)	3·5	4·75	3·5	6·5	6·5	6·5	5	1·5	7	4·75	7·25	4	7·25	5·5	5·25
$\frac{GA}{Control} \times 100$	44	48	35	87	81	100	59	17	94	56	76	67	81	69	65

was observed after the administration of 100 mg. of glycyrrhetinic acid, each rat being used as its own control.

The mean water retention of the whole group was 35% ; only one out of 14 rats showed no inhibition of diuresis.

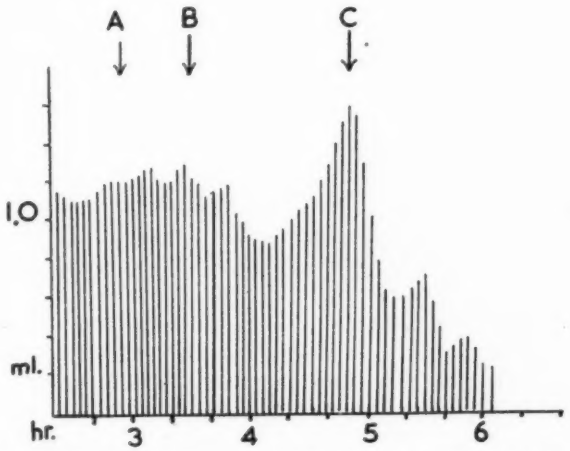

FIG. 2.—Effect of intravenous glycyrrhetinic acid on diuresis in a rat. Ordinate: Urine volume in ml./10 min. Abscissa: Time in hr. A: 0·05 ml. propylene glycol washed in with 0·1 ml. saline. B: same as A but containing 40 μg. glycyrrhetinic acid. C: Same as A but containing 150 μg. glycyrrhetinic acid.

(*c*) *Normal Dog.*—In control experiments without glycyrrhetinic acid, a mean rate of diuresis of 4 ml./min. was observed (Fig. 3). Glycyrrhetinic acid caused a depression of the rate of diuresis averaging 40% during the peak period. The volume of urine excreted over 3 hr. and 50 min. dropped by an average of 20% in the glycyrrhetinic acid experiments. In the one experiment with DOCA no such inhibition was observed.

To see whether the antidiuretic effect of glycyrrhetinic acid was due to a lowering of the blood pressure, the femoral vein of a 200 g. rat under urethane anaesthesia was cannulated and connected to a saline-filled burette ; the carotid artery was also cannulated and connected to a sensitive blood-pressure manometer. Propylene glycol produced a short-lived rise in blood pressure, and ethanol in the same dilution produced a smaller pressor effect. Glycyrrhetinic acid (0.4 mg. in 20% alcohol) certainly had no hypotensive effect ; it caused a rise in pressure similar to that due to the solvent.

Since the antidiuretic effect of glycyrrhetinic acid could not be attributed to a fall in blood pressure, attempts were made to discover what mechanisms were involved. Rats in which removal of the posterior lobe of the pituitary had been attempted were used, and their responses compared with those of conscious normal rats (Table V). In order to be able to do cross-over tests, the diuresis was followed in the unanaesthetized animal and the drug given by mouth.

TABLE V

DIURESIS IN RATS AFTER REMOVAL OF THE POSTERIOR LOBE OF THE PITUITARY

The rats had free access to water; urine collected over 18 hr.

Rat Number	Urine Secretion as % of Control				
	6th Day after Operation	1st Experiment*		2nd Experiment*	
		Before	After	Before	After
1	190	93	311	—	—
2	141	224	239	376	372
3	190	93	710	—	—
4	270	195	533	106	286
5	1,400	1,830	350	370	330
6	2,360	3,170	510	650	400

* Before and after the corresponding glycyrrhetinic acid experiments (Table VI).

(*d*) *Rats with Diabetes Insipidus.*—In Table V the degree of diabetes insipidus attained by the operated rats is presented. The values are calculated as percentage of urine secretion by non-operated controls of the same batch. The first values given are those for the sixth night after operation ; they are followed by the figures for the night preceding, and that following, each glycyrrhetinic acid test. The figures in this table show that the degree of diabetes insipidus resulting from the operation was not steady from day to day. On the other hand, all rats showed a manifest increase in diuresis maintained till the animals died or were killed several weeks after the operation.

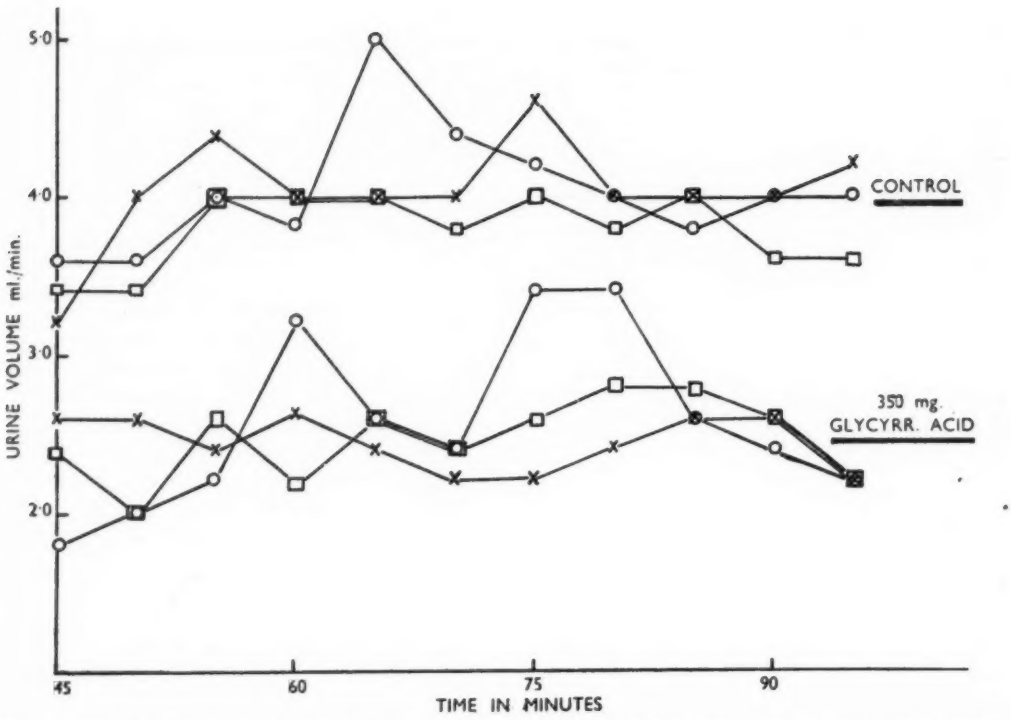

FIG. 3.—Five min. urine collections after two water loads of 250 ml. and 300 ml. Three tests and three control experiments carried out on the same dog. Ordinate: Urine volume in ml./ min. Abscissa: Time in min.

When glycyrrhetinic acid was administered to these rats, an inhibition of diuresis was observed (Table VI). This inhibition was comparable to that met with in non-diabetic rats. The mean water retention in these experiments was 41.5% compared with 35% in the non-diabetic rats.

To see whether delayed water absorption from the gastro-intestinal tract played any part in the reduction of water excretion, the method used by Heller and Smirk (1932) was employed to investigate the effect of glycyrrhetinic acid on water absorption.

Water Absorption from the Alimentary Canal

In the first series of experiments (Table VII) the weight of the gut, when expressed as percentage of body weight, was always higher after glycyrrhetinic acid than in the control rats. This was so whether the rats were killed 15 or 60 min. after the water load. No significant difference in the degree of water retention was observed between the two groups. Glycyrrhetinic acid can be detected in the stomach as a white precipitate 4½ hr. after administration.

In the second series of experiments, the dose of glycyrrhetinic acid was trebled and three groups,

TABLE VI

EFFECT OF ORAL GLYCYRRHETINIC ACID ON DIURESIS IN RATS WITH DIABETES INSIPIDUS

Urine collected over 2 hr. after a load of 12 ml. H_2O.

No. of rat	1	2		3	4		5		6		Mean
No. of experiment		A	B		A	B	A	B	A	B	
Control day (ml. urine)	8·75	8·5	7·0	10·5	3·0	2·75	10·75	9·5	10·0	6·75	7·75
After 100 mg. glycyrrhetinic acid (ml. urine)	7·75	6·5	6·25	6·0	1·5	0·75	5·5	6·0	5·5	2·0	4·78
$\frac{\text{Test day}}{\text{Control day}} \times 100$	88·5	76·5	89·0	57·0	50·0	27·0	51·0	63·0	55·0	30·0	58·5

TABLE VII

EFFECT OF ORAL GLYCYRRHETINIC ACID (GA) ON ABSORPTION OF WATER FROM THE GUT OF RATS

No. of Pair	Time After Giving Load (min.)	Wt. of Gut as % of Body Wt.		$\frac{b}{a} \times 100$*
		Control (a)	GA (b) 100 mg.	
1	15	8·0	8·9	111
2	15	8·6	9·6	112
3	15	9·0	9·4	105
4	15	7·4	8·6	116
5	15	8·3	10·0	120
	Mean	8·26	9·3	113
1	60	6·2	7·2	117
2	60	6·6	8·5	129
3	60	5·4	5·8	107
4	60	6·2	7·5	121
5	60	6·2	6·4	103
	Mean	6·12	7·08	115

* The differences between the mean of test and control rats are significant (P=0·01–0·001 after 15 min.; P=0·02–0·01 after 60 min.), provided the differences between each pair are used as the basis of the calculations.

killed at different time intervals, were employed. Each group consisted of ten rats, five receiving the acid and five acting as controls. As is shown in Table VIII and Fig. 4, the control groups give lower mean values than the test groups. The maximal difference in the second series was similar to that observed in the first series of experiments, where the dose of the acid given to each rat was only 100 mg. compared with 300 mg. given in the second series.

The mean gut weight, in both control and test animals, decreased gradually with time, indicating the gradual adsorption of the intestinal contents. The controls show the same kind of curve as that described by Heller and Smirk (1932); a rapid rate of absorption in the first ½ hr. after which the curve flattens out. In the test group, the same pattern is maintained with two differences; the first part of the curve is less steep and the whole curve is maintained at a higher level throughout.

The weights of the livers did not show any significant difference. Heller and Smirk found the liver to show the most marked increase in weight in response to hydration; in this experiment, however, the increase was not significant.

TABLE VIII

EFFECT OF GLYCYRRHETINIC ACID ON THE ABSORPTION OF WATER FROM THE GUT AND ITS RETENTION IN THE LIVER

Time since Water Load	No. of Rats in Group	Control		Glycyrrhetinic Acid	
		Mean Gut Wt. ± S.E.	Mean Liver Wt. ± S.E.	Mean Gut Wt. ± S.E.	Mean Liver Wt. ± S.E.
15 min.	10	12·6±0·54	4·8±0·41	13·8±0·84	5·1±0·25
30 ,,	10	9·7±0.32	4·6±0·29	12·4±0·67	4·7±0·25
75 ,,	10	9·1±0·43	4·8±0·16	10·2±0·58	4·9±0·1

When the rats received 100 mg. of the acid (Table VII), the mean weight of the gastro-intestinal tract was 6.12% of the body weight in the control group 1 hr. after the water load, and 7.08% in the glycyrrhetinic acid group. This is a difference of 0.96% of body weight or 19.2% of the water load.

Heller and Smirk (1932), in their original experiments, showed that the weight of the gut drops in the first hour and reaches a level between 6% and 7% of the body weight and that this level is maintained after that. They reported that absorption of a 5% load is completed 15–20 min. before the height of diuresis is reached. The 19.6% difference between control and glycyrrhetinic acid groups can, therefore, be taken as an estimation of the degree of water retention in the gut caused by 100 mg. of glycyrrhetinic acid.

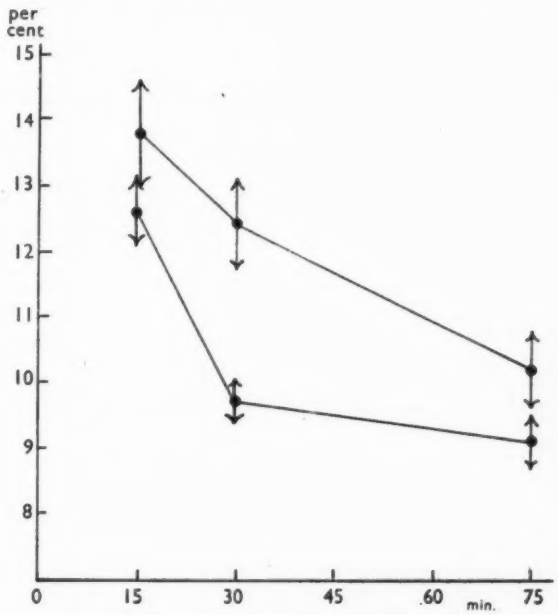

FIG. 4.—Effect of glycyrrhetinic acid on water absorption from the alimentary tract after a water load of 5% of body wt. Each point represents the mean wt. of the gut in five rats. The arrows show the standard error of the mean. Ordinate: Mean gut wt. as percentage of body wt. Abscissa: Time allowed for absorption in min. The upper curve represents the results of rats receiving 100 mg. glycyrrhetinic acid. The lower curve represents the results of control rats receiving the solvent only.

In the diuresis experiments reported in Table IV, the differences between control values and those following 100 mg. of the acid had a mean of 3.04 ml. or 25.3% of the load. Here, however, the load was almost double that used in the absorption experiments. The percentage of water retention in the gut is likely to be smaller with a bigger water load if the same dose of the acid is used. Therefore, it is unlikely that the delay in absorption of

water on giving glycyrrhetinic acid by mouth accounts for the whole 25.3% of the water retained.

To investigate further the role played by gastro-intestinal water retention in the anti-diuretic action of glycyrrhetinic acid the following experiment was carried out.

Albino rats (150 g.) were denied food for 24 hr. At 10 a.m. water was withdrawn and the animals divided into pairs of similar weight. At 12 noon, 0.2 ml. propylene glycol was injected intraperitoneally into one rat of each group. The other had 10 mg. of the acid dissolved in 0.2 ml. of propylene glycol. A water load of 12 ml. tap-water was given by stomach tube 10 min. later. The rats were transferred to metabolism cages and urine was collected for 150 min. At the end of the collection the rats were killed and their gastro-intestinal tracts dissected out. The contents of the caecum were emptied out and the gut weighed.

TABLE IX

EFFECT OF GLYCYRRHETINIC ACID ADMINISTERED INTRAPERITONEALLY ON DIURESIS AND WATER ABSORPTION

(Water load 12 ml.)

Group No.	Control		Glycyrrhetinic Acid	
	Urine % of Load*	Gut Weight % of Body Wt.	Urine % of Load*	Gut Weight % of Body Wt.
1	70	7·7	20	7·8
2	30	9·3	2·3	7·9
3	30	7·3	5	8·4
Mean	43	8·1	9·1	8·03

* Urine collection carried out over 150 min.

As shown in Table IX, 10 mg. of the acid intraperitoneally inhibited diuresis in the test rats. The water retained in these rats is not retained in the gut, as is evident by the identical weight of the gut in the control and test rats. In two of the rats, a minor degree of peritoneal effusion was noticed which, however, was not sufficient to account for the water retention.

DISCUSSION

Mineral Metabolism

The success reported by several workers (Groen, Willebrands, and Kamminga, 1951 ; Groen, Frenkel, and Kamminga, 1952 ; Card, Mitchell, Strong, Taylor, Tompsett, and Wilson, 1953 ; and Pelser, Willebrands, Frenkel, Heide, and Groen, 1953) in the treatment of adrenal deficiency with glycyrrhetinic acid and liquorice prompted the investigation of the mode of action of glycyrrhetinic acid on the salt metabolism of rats.

Under experimental conditions in which 1 μg. DOCA caused a significant degree of sodium retention, 400 μg. glycyrrhetinic acid caused no sodium retention, provided the collection of urine was continued long enough to allow urinary flow. A tendency to accelerate potassium excretion was only observed when a sizable potassium load was given.

While it may be justifiable to conclude that glycyrrhetinic acid does not behave like DOCA, as far as the salt metabolism of adrenalectomized rats is concerned, it was conceivable that, like Compounds B, E, or F of Kendall, which are only weak salt retainers, glycyrrhetinic acid may have a beneficial effect on adrenocortical deficiency. No such action on weight or survival time of adrenalectomized rats was, however, found.

Glycyrrhetinic acid, in doses of 3 mg./rat/day, failed to produce any significant change either in the serum sodium or potassium of adrenalectomized rats. This dose is equivalent to 600 mg. to a 60 kg. man. Pelser *et al.* (1953) found 60–100 mg. of the acid to be effective in Addisonian patients when given intramuscularly. Card *et al.* (1953) found 50 mg. doses q.i.d. to be effective in a patient suffering from Addison's disease. The dose employed in the reported rat experiments falls well within the effective range in terms of these human doses.

These findings, therefore, confirm the findings of Nelemans and Stamperius (1949) and Card *et al.* (1953) according to which glycyrrhetinic acid did not cause significant changes in the electrolyte balance or survival times of adrenalectomized rats.

The reported favourable effects of the acid in Addisonian patients in contrast to the lack of such an effect in rats suggest two possible explanations: (*a*) the drug may be metabolized in man in a different way, giving by-products which are active in this respect ; or (*b*) the mere water retention in Addisonian patients may have favourable effects under certain conditions.

Water Metabolism

In this series of experiments, it was possible to demonstrate the water-retaining effect of glycyrrhetinic acid in albino rats and in a dog.

In all the previous reports, the water retention by glycyrrhetinic acid has been related to its reported DOCA-like effect on mineral metabolism. Since no such effect was produced in adrenalectomized animals, another explanation was called for. It may also be emphasized that in many conditions DOCA has a diuretic, rather than an anti-diuretic, action (Gaunt, Birnie, and Eversole, 1949 ; Gaunt, 1943 ; Gellhorn and Ballin, 1946 ; Winter, 1952 ;

Kuhlman, Ragan, Ferrebee, Atchley, and Loeb, 1939 ; Winter and Ingram, 1943 ; Boss, Birnie, and Gaunt, 1949).

The mechanism of the anti-diuretic action of glycyrrhetinic acid appears to be a complex one. No marked change of the blood pressure was observed when the drug was administered intravenously, which suggested that low filtration pressure in the kidneys was not the cause of antidiuresis. The drug was effective in causing water retention in rats with diabetes insipidus during the height of the diabetic phase. The rate of water retention in these diabetic rats was comparable to the rate of water retention in intact rats under the same conditions. The posterior lobe of the pituitary does not therefore seem to play a role in this mechanism.

The drug, when given by mouth in doses identical with those used in the diuresis experiments, caused a significant degree of water retention in the gut. The percentage of water retained in the diuresis experiments, however, was somewhat higher than the percentage of water retained in the gut. That other mechanisms are involved is shown by the fact that, intraperitoneally, the drug in small doses caused a marked suppression of diuresis and no water retention in the gut. In addition, minute doses administered intravenously to rats anaesthetized with alcohol caused a marked depression of diuresis. In the sodium balance experiments, the drug was observed to cause water retention when administered subcutaneously.

By mouth, glycyrrhetinic acid seems to cause water retention in the alimentary canal as well as in the internal compartments of the body. The second effect may be due to the tubular reabsorption of water in the kidneys as suggested by Molhuysen, Gerbrandy, de Vries, de Jong, Lenstra, Turner, and Borst (1950).

The acid is precipitated in the stomach when introduced in propylene glycol and can still be detected there 4½ hr. after administration. This fact provides a possible explanation of the greater efficacy of parenteral as compared with oral administration.

Summary

1. The reported DOCA-like activity in man of liquorice extracts and glycyrrhetinic acid has been investigated in animals.

2. When these drugs were given to adrenalectomized rats no effect on the sodium balance was observed. Only during the period in which diuresis was suppressed was there a retention of sodium. The increase in potassium excretion was not significant unless the level of the extracellular potassium was highly elevated.

3. Prolonged treatment of adrenalectomized rats with glycyrrhetinic acid caused no significant improvement in the level of serum electrolytes, the body weight, or the survival time.

4. Given by any route, the acid had an antidiuretic effect. The posterior lobe of the pituitary is not involved in this action of the drug ; after parenteral administration, the effect is perhaps produced by a stimulation of the tubular reabsorption of water.

5. When given by mouth, the drug delays water absorption from the alimentary tract. The delay is sufficient to contribute to the water-retaining effect of the drug when administered orally.

6. It is concluded that the actions of the drug are different from those of DOCA.

I wish to express my gratitude to Professor J. H. Gaddum for his continued advice, and to Dr. M. Vogt, whose willing help and guidance made this work possible.

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ENZYMIC OXIDATION OF DERIVATIVES OF ETHYLENEDIAMINE

BY

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WITH AN ADDENDUM BY A. ALBERT

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(RECEIVED MARCH 24, 1955)

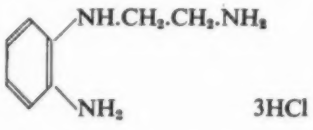
A number of derivatives of ethylenediamine, prepared recently in Dr. H. R. Ing's laboratory, have been examined as possible substrates of amine oxidases, and the results of this study are described in this paper.

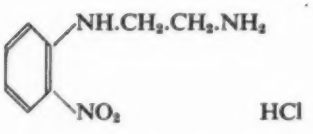
Ethylenediamine is a poor substrate of histaminase; it is oxidized neither by amine oxidase nor by spermine oxidase, two enzymes that will act upon diamines in which the two amino groups are more widely separated. The lack of affinity of ethylenediamine and other short-chain diamines, e.g., putrescine or cadaverine, for these enzymes must be due to a disturbing effect exerted by one amino group upon the reaction of the other amino group with the enzyme.

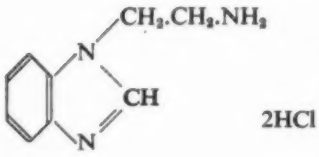
The action of amine oxidase, spermine oxidase, and histaminase on the compounds described below has been examined in this study.

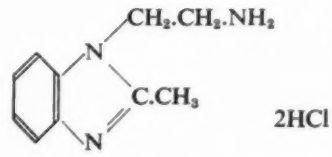
MATERIAL AND METHODS

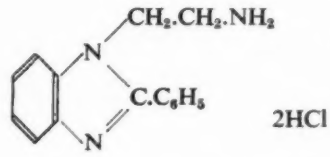
(a) *Compounds Studied.*—These were:

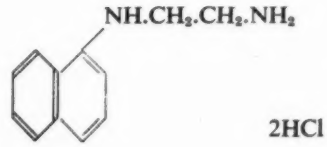
I  *N-o*-aminophenylethylenediamine trihydrochloride

II  *N-o*-nitrophenylethylenediamine hydrochloride

III  1-2′-aminoethylbenziminazole dihydrochloride

IV  1-2′-aminoethyl-2-methylbenziminazole dihydrochloride

V  1-2′-aminoethyl-2-phenylbenziminazole dihydrochloride

VI  α-naphthylethylenediamine dihydrochloride

For compounds I to V we are grateful to Drs. E. F. Rogers and R. Foster and to Mr. F. Jenkins; compound VI was a commercial product, obtained from Messrs. Roche.

(b) *Enzyme Preparations.*—These were similar to preparations commonly used in this laboratory. A fully dialysed preparation of either guinea-pig or rabbit liver (1 g. fresh tissue in a total volume of 2 ml.) in 0.02M-sodium phosphate buffer of *p*H 7.4 served as a source of amine oxidase. The histaminase preparation was an extract, in 0.067M-sodium phosphate buffer of *p*H 7.4, of an acetone-dried powder of pig kidney. Ox serum, fully dialysed against the same 0.067M-phosphate buffer, was used in the experiments on spermine oxidase.

RESULTS

Amine Oxidase.—All the compounds were tested with the preparations of both rabbit and guinea-pig liver. Compounds I, II, and III were found to be oxidized. That the oxidation was due to

amine oxidase is strongly supported by the finding that with each of these compounds the rate in the presence of sympatol was intermediate between the rate with the compound alone and sympatol alone.

Oxygen uptake, in competition with sympatol, also occurred with compound VI in the presence of rabbit liver extract, but no oxygen uptake occurred in the presence of guinea-pig liver extract.

Compounds IV and V were oxidized neither by rabbit liver nor by guinea-pig liver.

These results are summarized in Table I.

TABLE I

OXIDATION OF ETHYLENEDIAMINE DERIVATIVES BY RABBIT AND GUINEA-PIG LIVER

Substrate concentrations: 5×10^{-3}M.

The figures represent $\mu l.O_2$ consumed in the first 15 min. of incubation.

Compound	Rabbit Liver			Guinea-pig Liver		
	With Ethylenediamine Derivative	With Sympatol	With Both Amines	With Ethylenediamine Derivative	With Sympatol	With Both Amines
I	94	29	87	100	73	96
II	41	3	7	5	62	13
III	29	18	23	19	50	34
IV	0	39	26	0	52	32
V	0	39	17	0	59	46
VI	25	24	26	0	30	—

Spermine Oxidase (Table II).—Ox serum rapidly oxidized compounds I, II, and VI. The oxygen uptake in the presence of spermine and one of these three compounds was competitive and not additive, suggesting that one and the same catalyst was responsible for the oxidation of spermine and of compounds I, II, and VI.

TABLE II

OXIDATION OF ETHYLENEDIAMINE DERIVATIVES BY SPERMINE OXIDASE OF OX SERUM

Substrate concentrations: 5×10^{-3}M.

The figures represent $\mu l.O_2$ consumed in the first 30 min. of incubation

Compound	With Ethylenediamine Derivative	With Spermine	With Both Amines
I	37	97	65
II	34	97	46
III	7	97	5
IV	9	97	68
V	0	76	13
VI	27	44	32

With compounds III, IV, and V, oxygen uptake in the presence of ox serum was small and of doubtful significance, but these substances had a marked effect upon the rate of oxidation of spermine. This is clearly shown by the following experiment, in which the rate of oxidation of spermine in the presence of different concentrations of compound III was measured. In the first 30 min. of the reaction the oxygen uptakes were:

with 5×10^{-3}M spermine alone		71 $\mu l.$ O_2
plus 5×10^{-3}M compound III		1 „ „
„ 5×10^{-4}M	„ „	13 „ „
„ 5×10^{-5}M	„ „	39 „ „
„ 5×10^{-6}M	„ „	70 „ „

In other words, compound III acted as an inhibitor of spermine oxidase.

Histaminase.—None of the compounds examined (I to V) was oxidized by histaminase. Added in a concentration equimolecular to that of the substrate, 5×10^{-3}M cadaverine dihydrochloride, they all depressed the rate of oxidation; the inhibitions were between 50 and 90%.

DISCUSSION

The experiments described clearly indicate that the derivatives of ethylenediamine studied, when tested with amine oxidases, differ from the aliphatic parent compound. None of the substances was oxidized by histaminase. Several compounds were oxidized by amine oxidase, an enzyme that does not act upon ethylenediamine. The oxidation of compound I, which is a derivative also of *o*-phenylenediamine, by the liver preparations might be considered as due to cytochrome oxidase, but this is unlikely as it has been shown that the rate of oxidation in the presence of sympatol is competitive. For the other compounds tested, there is even less reason to doubt that the catalyst responsible for oxidation was amine oxidase.

The fact that the benziminazole derivatives substituted in position 2 by a methyl or a phenyl group were not oxidized by amine oxidase is possibly explained by steric factors, which prevent the attachment of the substrate essential for the occurrence of oxidation.

The specificity requirements of spermine oxidase are in many ways similar to those of amine oxidase, and it is therefore of interest that ox serum acted upon three of the ethylenediamine derivatives tested (I, II, and VI) and that the oxidation of spermine was strongly inhibited by the other compounds.

Solutions of compounds III to V were strongly acid; these compounds are dihydrochlorides, but each requires about one equivalent of alkali for neutralization. Compound I, which is a trihydrochloride, required about two equivalents of base for neutralization.

This observation suggested that these compounds differed from ethylenediamine in that they con-

tained only one strongly basic amino group which was ionized at the *p*H at which the experiments were carried out.

A sample of the benziminazole derivative (III) was therefore sent to Professor A. Albert, who has kindly determined the ionization constants of this compound. A report on his findings appears as an Addendum to this paper. His results allow us to conclude that compound III, and probably also all the other substances examined in this study, were present as mono-cations at the *p*H at which the experiments were carried out.

The experimental evidence thus strongly suggests that the degree of ionization is one of the factors which determines whether an amine is oxidized by amine oxidase or by histaminase. The ethylenediamine derivatives examined are substrates of amine oxidase because they carry, like monoamines, only one basic group, the free amino group at the end of the side chain. They differ from the aliphatic diamines which, at the *p*H of our experiments, are present almost fully as the di-cations (Schwarzenbach, 1933).

These considerations, which help us to understand the enzymic oxidation of diamines, must be more generally applicable. Graham and Tonks (1954) have recently studied the pharmacological properties of certain ethylenediamine derivatives, in which one of the two amino nitrogen atoms is directly attached to an aromatic ring. It seems safe to predict, therefore, that the authors were investigating compounds which, at the hydrogen ion concentration of the tissue, were present as singly charged compounds.

Summary

1. A number of derivatives of ethylenediamine have been examined as substrates of amine oxidases.

2. In contrast to ethylenediamine, some of these compounds were oxidized by amine oxidase and by spermine oxidase, but not by histaminase.

3. These results are interpreted as being due to differences in the degree of ionization of ethylenediamine and its derivatives.

One of us (M. L. C.) wishes to record his deep gratitude to Professor J. H. Burn, F.R.S., for unfailing help and encouragement during his stay in this laboratory.

ADDENDUM

By A. Albert

A 0.005M-aqueous solution of 1-2′-aminoethylbenziminazole dihydrochloride was potentiometrically titrated with 0.1N-potassium hydroxide, which was added in tenth-equivalent portions (at 20°), and the antilogarithms of the pK_a values averaged. Two pK_a values were thus found, 4.32 ± 0.02 and 8.24 ± 0.02. The value 4.32 refers to the nitrogens of the benziminazole-ring, which share the positive charge between them because of a resonance effect; the higher value belongs to the more basic β-amino-group on the aliphatic chain. Thus, at *p*H 7.4, about 90% of this substance is ionized, but this is entirely a mono-cation with the positive charge on the β-amino-group. The second basic group (4.32) is about a hundred times weaker than those of ethylenediamine (6.98) and histamine (6.03). At *p*H 7.4, only 4% of histamine is present as the di-cation, which appears to be the ionic species required by histaminase. Putrescine and cadaverine are entirely di-cations at *p*H 7.4.

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IN VIVO AND *IN VITRO* COMPARATIVE ASSAYS OF THE ANTICOAGULANT ACTIVITY OF DEXTRAN SULPHATE AND HEPARIN

BY

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(RECEIVED MARCH 18, 1955)

The recent demonstration by Ricketts (1952), Walton (1952) and Ricketts and Walton (1952), that dextran sulphate preparations of low molecular weight are both anticoagulant and non-toxic, raises the problem of the measurement of potency of semi-synthetic anticoagulants and of the choice of a suitable standard for assay.

Several attempts have been made to assay other semi-synthetic anticoagulants in terms of heparin (Schutz, 1941; Reinert and Winterstein, 1939; Foster, 1942; Kuizenga, Nelson and Cartland, 1943). We have also used heparin, and made some observations on the mode of action of heparin and dextran sulphate. In our hands the *in vitro* methods depending upon the inhibition of blood coagulation proved to be impracticable because the dose-response curves of heparin and dextran sulphate were not parallel. We accordingly devised an *in vivo* method for the comparison of different anticoagulants. This method has the advantage that, compared with *in vitro* tests, the conditions in which potency is measured are closer to those of clinical use. Moreover, since the dose-response curves are steep, high ratios of minimal anticoagulant doses give a useful approximate estimate of comparative potency, irrespective of differences of dose-response of the substances compared.

METHODS

International standard heparin was used throughout. The proposed international standard for dextran sulphate was obtained from the Department of Biological Standards, National Institute for Medical Research, London. The Russell viper venom was reconstituted from desiccated venom obtained from the Central Research Institute, Kasauli, India.

By paper electrophoresis we separated four fractions of Russell viper venom. One of these—the "coagulant fraction"—contained 80% of the total coagulant activity of the crude venom, behaved like a thrombokinase, and had no thrombin activity.

In vitro *Assays.—In vitro* assays were made by the prothrombin-time method of Quick, as modified by Meyer, Piroue, and Odier (1952) using oxalated human plasma; 9 ml. venous blood, collected in a silicone-coated syringe, was immediately mixed with 1 ml. 0.1N-sodium oxalate and centrifuged for 10 min. at 2,800 r.p.m. at 15° C. The plasma was kept at room temperature and used within 3 hr. In one series of tests, coagulation was induced by thromboplastin (Difco) and $CaCl_2$; in another series by Russell viper venom and $CaCl_2$. In a few tests, in which guinea-pig plasma was used, the results were similar to those with human plasma, but were less consistent.

The standard heparin was dissolved in 0.85% saline at a concentration of 10 μg./ml.; the dextran sulphate in saline at a concentration of 81.5 μg./ml. Bacto-thromboplastin was added to saline at 50–55° C. to make a 1.875% (w/v) concentration and insoluble material removed by centrifugation; it was kept at 37° C. until used. The dried Russell viper venom was dissolved in saline, and a crystal of thymol added. About 7% of the dried material was insoluble and was removed by centrifugation; the remainder made up in saline, with thymol, to contain 0.1 mg. venom/ml.

Two assay techniques were used: (*a*) *With thromboplastin.* Plasma (0.1 ml.) was added to 0.2 ml. of graded concentrations of the dextran sulphate or of the heparin. The mixtures were kept at 20° C. for 10 min. and thereafter at 37° C. After 1–2 min., first 0.1 ml. thromboplastin and then 0.1 ml. 0.025N-$CaCl_2$—both at 37° C.—were added and mixed by inversion. The formation of a clot was detected by slowly moving a glass hook up and down the column of fluid. (*b*) *With viper venom.* The procedure differed from that with thromboplastin only in that the venom and the $CaCl_2$ were added simultaneously in a volume of 0.2 ml. This step was necessary because the venom initiated coagulation even in the absence of the calcium salt.

In vivo *Assays.*—As in the *in vitro* tests, Russell viper venom solutions were freshly prepared. The electrophoretically purified venom, in thymolized saline, was tested immediately *in vitro* for thrombokinase activity against a standard solution of total

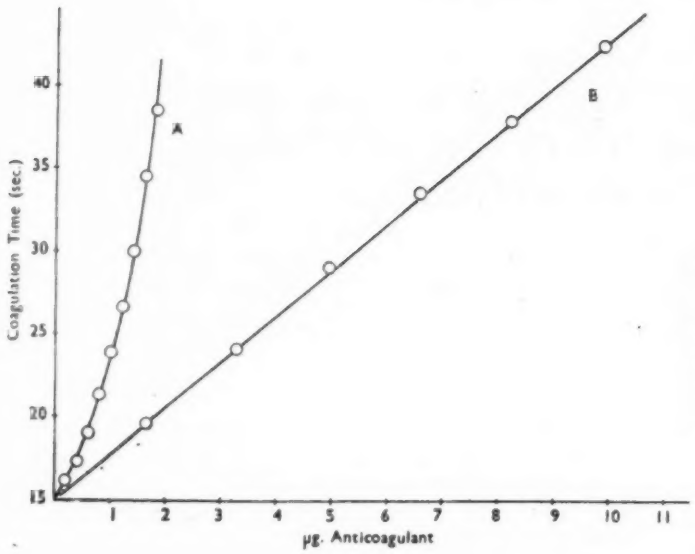

FIG. 1

FIG. 1.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Coagulation time with thromboplastin-treated plasma plotted against dose of anticoagulant. Graded concn. of anticoagulant in 0.2 ml. volumes added to 0.1 ml. fresh oxalated human plasma, held for 10 min. at 20° and 2 min. at 37°; 0.1 ml. 1.875% thromboplastin and 0.1 ml. 0.025N-$CaCl_2$ successively added, and the mixtures agitated with a glass hook at 37° C. until clotting occurred.

FIG. 2.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Experiment as in Fig. 1, except that log. coagulation time is plotted against dose.

FIG. 3.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Coagulation time with Russell-viper-venom-treated plasma, plotted against dose of anticoagulant. Graded concn. of anticoagulant in 0.2 ml. volumes added to 0.1 ml. fresh oxalated human plasma, held 10 min. at 20° C. and 2 min. at 37° C.; and a mixture of 0.1 ml. 0.025N-$CaCl_2$ and 0.1 ml. of 0.1 mg./ml. venom added, and the final mixtures agitated with a glass hook at 37° until clotting occurred.

FIG. 4.—Anticoagulant activity of heparin (A) and dextran sulphate (B). Experiment as in Fig. 3, except that log. coagulation time is plotted against dose.

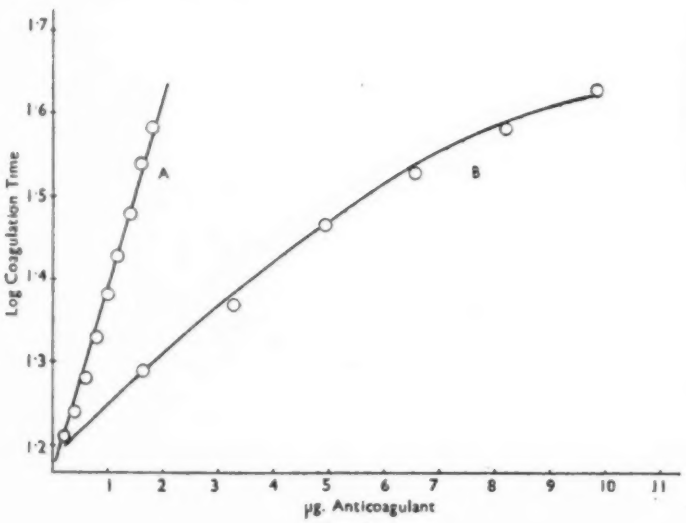

FIG. 2

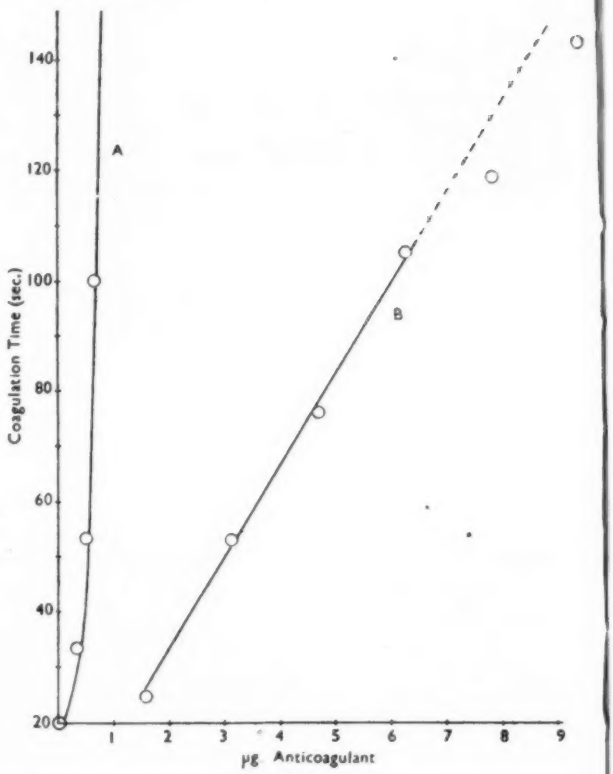

FIG. 3

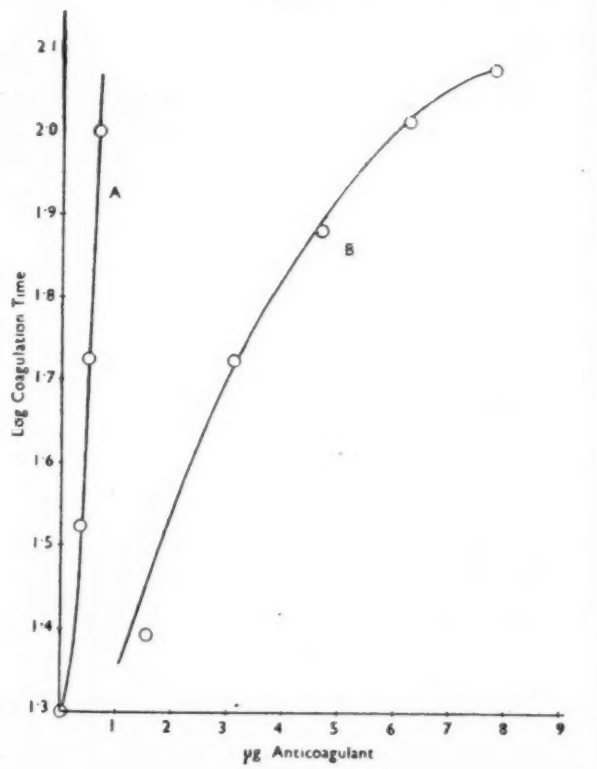

FIG. 4

Russell viper venom, and immediately used for injections.

The mixture of anticoagulant (heparin or dextran sulphate) with crude or purified Russell viper venom was made up to 5 ml. with physiological saline, allowed to stand for approximately 10 min. at room temperature, and slowly injected into an ear vein of a conscious rabbit over 2 to 3 min., carefully avoiding any subcutaneous leak. The animals were observed closely during the subsequent hours. Autopsies were usually carried out immediately after respiration had stopped. Often the heart was still beating or fibrillating. The cavities of the heart and the lumen in large vessels, particularly the pulmonary vessels, were searched for the presence of clots.

Results

In vitro *Assays*

With either bacto-thromboplastin or Russell viper venom, the relation of dose of heparin to coagulation time was curvilinear (Fig. 1A); with respect to the logarithm of the coagulation time it was linear (Fig. 2A). The dose of dextran sulphate on the other hand was linearly related to the coagulation time; its relationship to the logarithm of the coagulation time was curvilinear (Figs. 1B and 2B). For a comparison of activity it would be possible to select an arbitrary coagulation time—for example 30 sec.—and compare the doses of the two substances which correspond to this response. On this basis, we found that dextran sulphate had about one-third the activity of heparin. The invalidity of this method is evident when another period is chosen as a response level. Thus at 40 sec. the ratio of activities is 1:9. Similar discrepancies were found when the logarithm of the dose of heparin was used as abscissa. Similar results were obtained with Russell viper venom (Figs. 3 and 4).

In other words, since the dose-response curves are not linear or parallel, neither the orthodox assay nor the slope-ratio assay (Finney, 1952) are applicable. The discrepancies in the dose-response curves of the two substances indicate that we are dealing with fundamentally different substances, thus violating the hypothesis of similarity (Finney, 1952) upon which valid assay is based.

In vivo *Assays*

The results are summarized in Table I. Rabbits receiving approximately 0.2 mg. of Russell viper venom alone, or its coagulant fraction, died within ½ to 3 min.

Post mortem there was a massive clot in the right heart cavities and thrombosis in large pulmonary vessels; the lungs were pale yellow and ischaemic.

Crude Venom (Table I, A).—In the test rabbits there was a certain critical weight ratio of anticoagulant to venom below which both heparin and dextran sulphate failed to inhibit the rapid coagulation of blood by Russell viper venom. The test animals died almost as quickly as the controls and the post-mortem picture was similar. Above this critical ratio, the anticoagulant inhibited the coagulant action of Russell viper venom and thus prevented immediate death. The animals survived the injection without apparent discomfort. Later, however, they developed progressive signs of general intoxication together with, after 3 to 4 hr., intense oedematous infiltration of the eyelids. They died within 3 to 20 hr.

Post mortem, these animals had lung oedema with extensive zones of hyperaemia surrounding haemorrhagic foci and, usually, congestion of most other organs. However, there were no clots in the chambers of the heart or in the large vessels. The delayed death appeared to be due to toxic components other than the coagulant fraction which are not inhibited by heparin or dextran sulphate. This view is consistent with the fact that repeated injections of heparin up to a total of 5 times the anticoagulant dose fail to prevent the later death.

When the minimum amount either of dextran sulphate or of heparin was used to neutralize the

TABLE I

THE ANTICOAGULANT ACTIVITY OF HEPARIN AND DEXTRAN SULPHATE IN RABBITS TREATED WITH RUSSELL VIPER VENOM

	Anti-coagulant	Rabbit Wt. (g.)	Dose of Venom (mg.) *a*	Dose of Anti-coagulant (mg.) *b*	Venom: Anti-coagulant Ratio (*a* : *b*)	Survival Time
A Crude venom	None	920	0·2			2 min.
		1,515	0·22			1·5 ,,
		1,320	0·2			3 ,,
	Standard heparin	980	3·0	6·0	1 : 2	2½–3½ hr.
		1,150	1·5	3·0	1 : 2	2½–3½ ,,
		2,250	0·6	1·2	1 : 2	12–20 ,,
		920	0·6	0·6	1 : 1	6 ,,
		1,070	0·6	0·6	1 : 1	10 ,,
		980	0·6	0·3	1 : ½	2½–3½ ,,
		1,050	0·6	0·3	1 : ½	1½ ,,
		1,200	0·6	0·3	1 : ½	3 min.
		1,290	0·3	2·8	1 : 9	12–24 hr.
	Standard dextran sulphate	1,200	0·3	81·5	1 : 272	2¼ hr.
		1,300	0·6	12·0	1 : 20	2 ,,
		1,090	0·6	9·0	1 : 15	2–3 min.
		2,460	0·6	6·0	1 : 10	2 ,,
		2,720	0·6	2·4	1 : 4	3 ,,
B Purified venom	None	1,480	0·15			>14 days
		1,440	0·2			3 min.
		1,140	0·35			2 ,,
	Standard heparin	1,470	0·8	1·0	1 : 1·25	>14 days
		1,140	0·35	0·35	1 : 1	>14 ,,
	Standard dextran sulphate	1,120	0·8	35·4	1 : 44·3	>14 days
		960	0·35	7·0	1 : 20	>14 ,,
		1,300	0·35	3·5	1 : 10	3 min.

immediate coagulant effect of the venom (venom : heparin = 1 : 0.5 ; venom : dextran sulphate = 1 : 20), death sometimes resulted from a late and progressive thrombus formation. In each of three rabbits so dying, a thrombus was found in the portal vein.

Our results agree with those of Ahuja, Veeraraghavan, and Menon (1946, 1947) as regards the weight ratio of heparin necessary to prevent death, and show clearly that the semi-synthetic anticoagulant dextran sulphate, if used in sufficient amount, has a total *in vivo* effect similar to that of heparin.

Purified Venom : Coagulant Fraction Isolated by Paper Electrophoresis (Table I, B).—As with crude venom, there was a certain critical ratio below which both heparin and dextran sulphate failed to prevent immediate death. Test rabbits died with the same signs and lesions as the controls. Above this ratio (for heparin 1 : 1 ; for dextran sulphate 1 : 20) they survived the immediate " coagulation " death ; indeed they survived for more than two weeks. Unlike those tested with crude venom, they did not show any signs of subsequent delayed intoxication, including the oedema of the eyelids —a useful criterion of the purity of the coagulant fraction of the viper venom.

Discussion

The data in Table I provide no basis for a comparison of the dose-response in heparin-treated and dextran-sulphate-treated animals. Nevertheless, it is clear that both with heparin, where the doses increase by twofold steps, and with dextran sulphate, where the dose increments are arithmetic, in steps of 3 mg., the response changes sharply from the acute to the delayed death.

With such steep dose-response curves, we may therefore take acute death as an index and directly compare minimal protective doses of the two anticoagulants. Potency ratios so determined have an added advantage over those obtained by *in vitro* methods, in that the conditions of the *in vivo* test are closer to those in clinical use of the substances, and so give a better indication of clinical efficacy.

From the results of these *in vivo* experiments, it is clear that, like heparin, dextran sulphate inhibits the coagulant action of either crude or purified Russell viper venom, whether it is used as a crude venom or as a purified fraction ; and that dextran sulphate of a potency equal to that of the proposed standard has, weight for weight, one-twentieth to one-fortieth of the anticoagulant efficacy of the international standard heparin.

Summary

1. The *in vitro* test for anticoagulant activity, based on Quick's method of measuring prothrombin time, does not provide a biometrically valid measure of the anticoagulant potency of dextran sulphate in terms of heparin.

2. Dextran sulphate and heparin both inhibit the coagulant action of Russell viper venom thrombokinase, *in vitro* and *in vivo*.

3. *In vivo*, intravenous Russell viper venom kills rabbits within a few minutes. When this acute death is prevented by previous mixing of the venom with either heparin or dextran sulphate, the animals die within 3 to 20 hr., owing to toxic substances other than the coagulant. These other toxins can be removed by paper electrophoretic fractionation of the crude venom.

4. An *in vivo* test, using Russell viper venom or the purified coagulant fraction, is described for the comparison of anticoagulant activity of heparin and anticoagulants of the dextran sulphate type. According to this test, the dextran sulphate constituting the proposed international standard is 20 to 40 times less potent than the international standard heparin.

We wish to thank Dr. W. L. M. Perry, Director of the Department of Biological Standards, National Institute for Medical Research, London, for the international standard heparin and the proposed international standard dextran sulphate. Our thanks go also to Dr. C. R. Ricketts and Dr. K. W. Walton, who kindly put at our disposal samples of dextran sulphate preparations for clinical use.

We are also indebted to the Scientific Department of Hoffman–La Roche for Liquemin and fibrinogen used in these experiments.

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THE METABOLISM OF ^{32}P-LABELLED TRIETHYLENE-PHOSPHORAMIDE IN RELATION TO ITS ANTI-TUMOUR ACTIVITY

BY

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From the Christie Hospital and Holt Radium Institute, Manchester

(RECEIVED MARCH 28, 1955)

Ethyleneimines such as triethylenephosphoramide (TEPA, I) and triethylenemelamine (TEM, II) (p. 325) exert remarkable inhibitory effects on the growth of some experimental tumours without seriously impairing the health of the host animal. Total regression of established and actively growing tumours may be achieved (Sugiura and Stock, 1952a and b; Crossley, Allison and Muenzen, 1952; Sugiura and Stock, 1955). TEPA was most effective against the rat tumours R 39 and Jensen sarcoma, less so against the Flexner-Jobling carcinoma and Walker carcinosarcoma 256, and had no action on the Murphy-Sturm lymphosarcoma (Sugiura and Stock, 1955). The Walker carcinosarcoma has been shown to undergo inhibition and then regression, but it later recurs and is then refractory to further treatment (Jackson, 1954). A study of the metabolism of TEPA labelled with radioactive phosphorus was undertaken in the hope of obtaining information about the mode of action of these compounds and of correlating tumour susceptibility with drug fixation in neoplastic tissue.

METHODS

Preparation of Triethylenephosphoramide-^{32}P.—The method used was based on that described by Bestian (1950) for the preparation of the unlabelled compound. Phosphorus oxychloride (3 g.) labelled with ^{32}P (20 mc.) was diluted to 15 ml. with dry benzene and added slowly to a well-stirred mixture of ethyleneimine (2.52 g.) and triethylamine (5.92 g.) in dry benzene (20 ml.). $POCl_3 + 3NH(CH_2)_2 + 3N(C_2H_5)_3 \rightarrow PO[N(CH_2)_2]_3 + 3N(C_2H_5)_3.HCl$. Vigorous stirring was continued for an hour before removal of the precipitated hydrochloride. The filtrate was concentrated to 2–3 ml. *in vacuo* and centrifuged to remove a small amount of solid. Aliquots of the clear supernatant fluid were cautiously distilled *in vacuo* in a micro-distillation apparatus. If the oil is heated above 130° it is liable to decompose violently. The distillate (b.p. 90° C. at 0.3 mm.) was collected on a central tube cooled with solid CO_2. It crystallized as a colourless hygroscopic solid which was stored in sealed containers at −20° C.

The purity of the labelled compound was assessed by ascending paper chromatography on Whatman No. 1 paper. Two solvent systems, water-saturated *n*-butanol and 80% ethanol, were used, and gave single, well-defined peaks with R_F values of 0.57 and 0.8 respectively.

Animal Techniques.—The distribution of radioactivity after a single injection of labelled TEPA was studied in rats of an American Wistar strain (100–150 g.) implanted with the Walker carcinosarcoma 256, or bearing a resistant variety of the same tumour developed by treatment with TEM (Jackson, 1954). The distribution after repeated injections (five consecutive daily doses) was studied in rats of the same strain implanted with the Jensen sarcoma. Freshly prepared aqueous solutions of labelled TEPA (specific activity 2,000 counts/min./μg.) were injected intraperitoneally at a dose of 1.0 mg./kg. No significant toxic effects occurred after five daily injections of this dose. Groups of animals were killed with ether at selected times. Blood was removed by ventricular puncture and diluted with 3.8% sodium citrate solution. Various tissue samples were then removed, care being taken to avoid cross-contamination during dissection. In each experiment a zero time was selected and all estimates of radioactivity were corrected to this time. As a standard procedure in the digestion of solid tissues for liquid counting, each weighed sample was treated with 2–3 ml. of lithium hydroxide solution (10% lithium hydroxide in 20% ethanol) and diluted to 10 ml. with water. Estimations of radioactivity were made in a liquid counter tube with an efficiency of about 7% for ^{32}P.

In determining the percentage excretion of the administered activity urine was collected from a group of five animals. Small amounts of urine were collected on a sheet of plastic cloth placed immediately beneath the cage. By feeding the animals with milk the degree of contamination of urine with faeces was greatly diminished. Aliquots of the urine (about 25 μl.) were chromatographed directly.

RESULTS

Anti-tumour Activity.—The inhibitory effect of TEPA on the growth of the Walker carcinosarcoma (5-day-old tumours) and its resistant form is shown in Table I. Six daily doses of labelled TEPA (2 mg./kg., i.p.) commencing 24 hr. after implantation of the Walker tumour resulted in complete inhibition for nearly three weeks. The tumour then grew in most animals. This effect is similar to that previously described for TEM (II) on the same tumour (Jackson, 1954). However, 6-day-old Jensen rat sarcomata, similarly treated, regressed completely (Table I) and there was no recurrence even after nine months.

Tissue Distribution After a Single Injection of Labelled TEPA.—Two groups of rats, implanted with the normal Walker tumour and its resistant variety respectively (tumour weights about 10 g.), were given the radioactive drug (1.0 mg./kg.). Samples of blood, plasma, spleen, and tumour all showed a rapid clearance of the drug in the first 4 hr. (Table II).

No significant difference was detected in the distribution of radioactivity in animals bearing the two varieties of Walker tumour. The mean tissue levels in a group of five normal rats at 24 hr. were 0.16%, 0.03%, and 0.21% of the administered activity/g. of blood, plasma, and spleen respectively. These values were similar to those of the tumour-bearing rats at the same time. Most of the administered radioactivity (80–90%) was present in urine collected during the first 24 hr. after injection.

Tissue Distribution after Repeated Injection of Labelled TEPA.—Rats bearing the Jensen sarcoma (tumours about 10 g.) were given five daily doses of labelled TEPA (1.0 mg./kg., i.p.). The tissue radioactivity was measured 24 hr. after the final dose (Table III), and again no appreciable retention of radioactivity was found. At the same time, the very low plasma radioactivity compared with that of the red cells and tissues suggested

TABLE I

THE INHIBITORY EFFECT OF TEPA (6 DAILY DOSES, 2 MG./KG., I.P.) ON THE GROWTH OF THE WALKER CARCINOSARCOMA 256, A RESISTANT FORM OF THE SAME TUMOUR, AND THE JENSEN SARCOMA

In all groups ten animals were used. The times given refer to the number of days after implantation of the tumours.

	Walker Tumour		Resistant Walker Tumour		Jensen Sarcoma	
	Controls	Treated	Controls	Treated	Controls	Treated
Day treatment began	—	4	—	4	—	6
Day experiment ended	11	11	11	11	21	21
Tumour wt. (g.)	28	1·8	39	22	22	0
	24	1·2	31	18	14	0
	22	1·0	23	13	12	0
	22	0·9	23	13	12	0
	21	0·9	21	12	11	0
	20	0·7	20	11	9·7	0
	18	0·5	20	7·5	9·3	0
	17	0·5	19	2·8	3·3	0
	14	0·4	17	2·8	0	0
	10	0·1	6·0	0·5	0	0

TABLE III

THE ^{32}P CONTENT OF VARIOUS TISSUES FROM RATS BEARING THE JENSEN SARCOMA (TUMOUR WT. ABOUT 10 G.) 24 HR. AFTER FIVE DAILY INJECTIONS OF LABELLED TEPA (1.0 MG./KG., I.P.)

Each value is the mean of five samples, the range being indicated in parentheses

	Counts/min./g.	% of Total Dose/g. Tissue
Blood	293 (270–322)	0·020
Plasma ..	18 (11–27)	0·001
Kidney ..	461 (432–494)	0·031
Liver	642 (605–700)	0·043
Spleen	546 (530–614)	0·036
Muscle ..	215 (177–240)	0·014
Testis	176 (134–198)	0·012
Tumour ..	330 (305–347)	0·022
Fat	108 (44–186)	0·007

TABLE II

TISSUE DISTRIBUTION OF RADIOACTIVITY AFTER ONE INJECTION OF LABELLED TEPA (1.0 MG./KG., I.P.)

Two groups of rats were implanted with the Walker carcinosarcoma and its resistant variety respectively. Each value is the mean of 5 samples, the range being indicated in parentheses. Results are expressed as the percentage of administered dose present/g. of tissue

	Hr. after Administration				
Walker tumour	0·5	1·5	4	24	48
Blood	0·78 (0·72–0·81)	0·50 (0·47–0·53)	0·18 (0·16–0·19)	0·11 (0·09–0·12)	0·08 (0·06–0·11)
Plasma	0·77 (0·74–0·79)	0·49 (0·36–0·64)	0·19 (0·14–0·25)	0·01 (0·005–0·017)	0·01 (0·008–0·013)
Spleen	0·69 (0·63–0·76)	0·49 (0·48–0·50)	0·23 (0·19–0·28)	0·20 (0·18–0·23)	0·12 (0·11–0·18)
Tumour	0·72 (0·68–0·75)	0·52 (0·49–0·57)	0·24 (0·17–0·27)	0·15 (0·13–0·22)	0·08 (0·07–0·11)
Resistant Walker tumour	0·5	1·5	3·5	12	24
Blood	0·69 (0·51–0·82)	0·45 (0·36–0·50)	0·18 (0·14–0·21)	0·08 (0·06–0·10)	0·11 (0·09–0·13)
Plasma	1·16 (0·65–1·50)	0·71 (0·54–0·76)	0·15 (0·10–0·18)	0·01 (0·008–0·013)	0·01 (0·006–0·013)
Spleen	0·66 (0·48–0·78)	0·49 (0·41–0·59)	0·23 (0·18–0·26)	0·21 (0·19–0·26)	0·21 (0·17–0·26)
Tumour	0·63 (0·44–0·76)	0·49 (0·41–0·54)	0·21 (0·14–0·23)	0·12 (0·07–0·16)	0·14 (0·10–0·16)

that some reaction of the drug with cellular components had occurred. Tumours fractionated chemically by the method of Davidson and Smellie (1952) showed no specific localization in particular cellular components. Thus, in the Jensen tumours referred to above the distribution of radioactivity was as follows: trichloroacetic acid soluble material, 32%; lipoids, 23%; nucleic acids, 25%; proteins, 20%. Crystallization of the haemoglobin from the blood samples and separation of the pigment into haematin and globin according to the method of Anson and Mirsky (1930) showed that over 90% of the ^{32}P was associated with the protein fragment. No such fixation of radioactive phosphorus occurred after the administration of repeated doses of labelled phosphate.

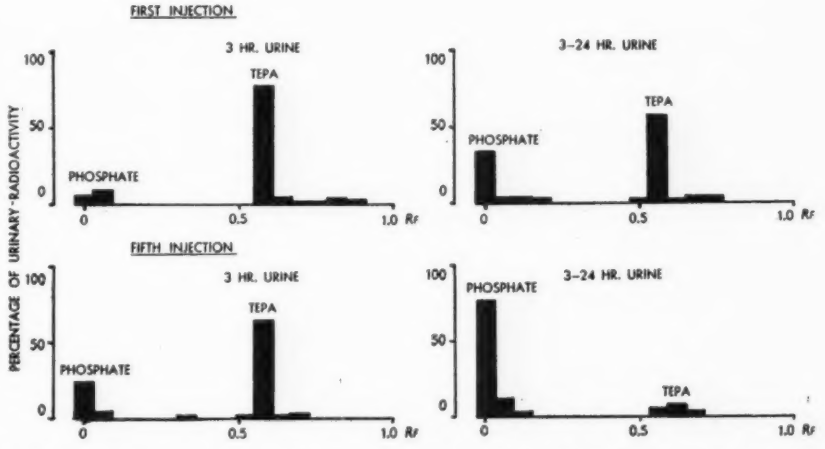


FIG. 1.—Chromatograms showing the proportions of phosphate and TEPA present in urine from rats bearing the Jensen sarcoma. Direct chromatography of samples collected 3 hr. and 24 hr. respectively after the first injection (upper graphs), and fifth injection (lower graphs), of radioactive TEPA (1.0 mg./kg., i.p.). The solvent system was water-saturated butanol. The increase in the proportion of inorganic phosphate in the 3–24 hr. urine is due to its slow excretion compared with unchanged TEPA. This is most apparent in urine collected 3–24 hr. after the 5th injection.

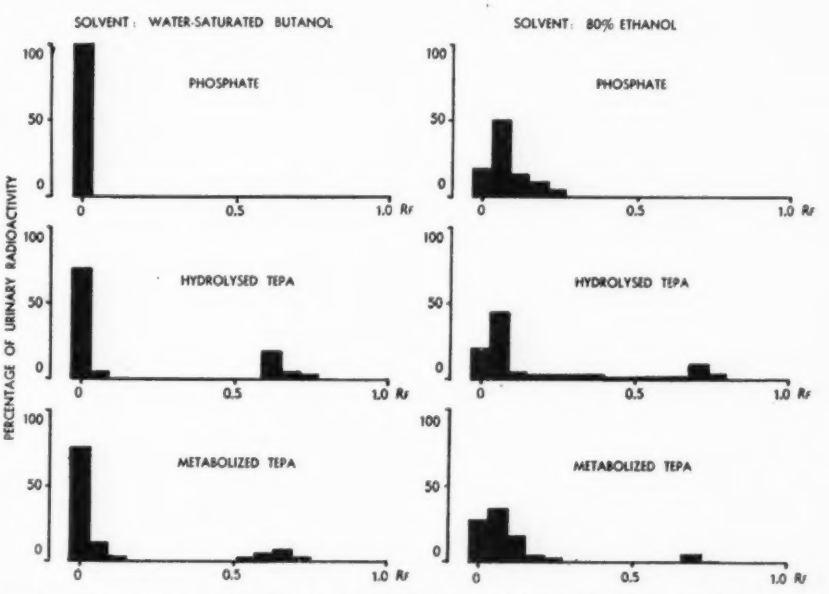


FIG. 2.—Chromatograms showing the similarity between labelled phosphate and the *in vivo* and *in vitro* breakdown products from radioactive TEPA. Hydrolysed TEPA was prepared by heating an aqueous solution (1 mg./ml.) at 95° for 24 hr. The metabolic components were present in rat urine collected 24 hr. after the last of a series of five doses of labelled TEPA (1.0 mg./kg., i.p.)

Metabolism of TEPA in Rats.—The chromatographic distribution of ^{32}P in the urine of animals given labelled TEPA was determined. Preliminary investigation of 24 hr. urine samples from treated animals bearing Walker tumours had shown that urine could be satisfactorily chromatographed with the solvent mixtures used for assessing the purity of labelled TEPA. These experiments had also indicated that a substantial portion of the drug was excreted unchanged. This was investigated more fully in animals with Jensen sarcomata during a course of 5 daily injections of the labelled drug. Urine was collected 3 and 24 hr. after each injection. Samples of the urine were chromatographed immediately. In 3 hr. samples 50–70% of the radioactivity was from unchanged drug, the rest of the radioactivity being located near the starting line (Fig. 1). Chromatograms of the 3–24 hr. urine samples showed a relative increase in the amount of metabolized phosphoramide which, in the last specimen, comprised 90% of the activity (Fig. 1).

The deterioration of an aqueous solution of the labelled TEPA was followed chromatographically. The product closely resembled the metabolite

found in urine. In view of the similar location of phosphate on chromatograms, the hydrolysis product from TEPA, and its principal urinary metabolite, appears to be inorganic phosphate (Fig. 2). Colorimetric estimations on aqueous solutions of TEPA also showed a slow liberation of inorganic phosphate.

Discussion

Cytotoxic agents like ethyleneimines and nitrogen mustards are believed to exert their pharmacological action by chemical interaction (alkylation) with unknown cell components vital to cell division. One is impressed by the emphasis laid on the chemical reactivity of these compounds. Thus Skipper, Bennett, and Langham (1951) using ^{14}C-labelled methyl-*bis*-(2-chloroethyl)amine found that after 24 hr. 80% of the administered radioactivity remained widely distributed and fixed throughout the tissues. Nevertheless, brief reference has been made to the absence of any specific localization of TEM (II) labelled with ^{14}C in the triazine ring (Skipper, 1953). Recently a more detailed account of the metabolism of this labelled compound in normal and tumour-bearing mice has appeared (Nadkarni, Goldenthal, and Smith, 1954). Following a single large dose (2 mg./kg.) about 80% was excreted in 24 hr., and chromatographic examination of the urine showed that no unchanged drug was present. The tissue radioactivity was widely distributed, and again no specific localization was observed. Chromatographic analysis of the 24 hr. urine revealed only one radioactive metabolite, which was identified as cyanuric acid (III). The presumption is that the ethyleneimino-groups were removed by a metabolic process and it was suggested that the biological activity of TEM might be related to a small portion of intact drug retained within the body. The rapid elimination of this drug is in agreement with previous experiments which suggested that its effect is short-lived, like that of the nitrogen mustards. Thus Goldberg and Schoenbach (1951) found that simultaneous injection of TEM and inoculation of mice with tumour inhibited the growth of the latter, whereas injection of the drug one hour before inoculation failed to hinder development of the tumour.

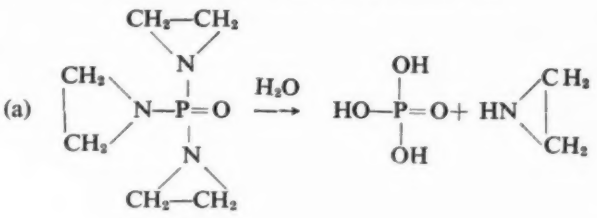
TEPA has been previously labelled with radioactive phosphorus and its distribution in rats examined (Crossley, Allison, Wannemacher, Migliarese, Parker, Kuh, Seeger, and Partridge, 1953). It was administered in a single large dose (about 25 mg./kg., s.c.) to normal rats and to rats bearing the Flexner-Jobling carcinoma, a susceptible tumour. Various tissues were examined up to 30 hr. after the dose, but no noteworthy selective concentration in normal or tumour-bearing rats was observed. About 65% of the radioactive dose was accounted for in the urine in 24 hr. In the plasma, 2 hr. after injection, the majority of the radioactivity (60%) was found by these authors to be precipitated with the globulin fraction, but no other data on the distribution in blood were given. No criteria of the purity of the TEPA used were published.

In earlier experiments of the present series, using TEPA known to be partially polymerized, a large fraction of the plasma radioactivity was also precipitated with the proteins. This did not occur in subsequent experiments when pure, labelled TEPA was used, although the tissue distribution pattern was in substantial agreement with the data of Crossley *et al.* (1953). The radioactive compound used in these later experiments was also shown to be pharmacologically active after the tracer studies had been completed, when it produced inhibition and temporary regression of the Walker tumour. These points are stressed since the small-scale preparation of pure radioactive TEPA is difficult because of its tendency to polymerize when distilled. Paper chromatography was found to be a good method of establishing its purity.

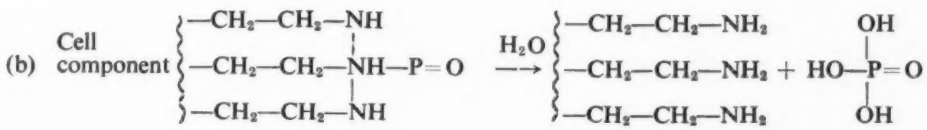
The precipitous fall in plasma radioactivity (Table II) and the recovery of over 80% of the dose in the 24 hr. urine show that TEPA is rapidly excreted. In all the tissues examined the amount of ^{32}P was greatest in the first sample ($\frac{1}{4}$ hr.), after which it fell rapidly at rates comparable to that in plasma. This correlates with the high proportion of unchanged radioactive TEPA present in urine, especially in the early stages (Fig. 1). This latter observation is in sharp contrast with the behaviour of labelled TEM which is completely metabolized in the mouse (Nadkarni *et al.*, 1954).

The idea that ethyleneimines are generally highly reactive alkylating agents under physiological conditions is not favoured by our results, which also suggest that only a small proportion of the drug produces the anti-tumour effect. It seems likely that the influence of the drug is exerted on a highly susceptible process in a sensitive tumour, for only a minute part of the dose is retained and there is no selective localization of radioactive material. Chromatographically, the only metabolite found in urine appears to be inorganic phosphate (Fig. 2), which might be produced by

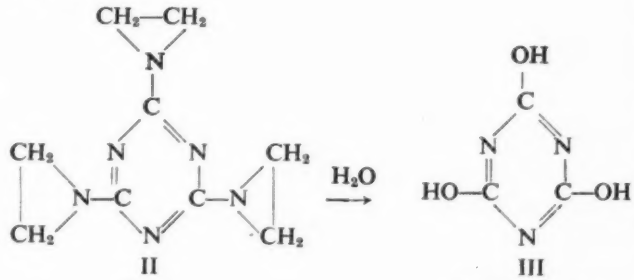
simple hydrolysis (a) or subsequent to reaction of the compound with tissue components (b):



I
TEPA



The *in vivo* production of inorganic phosphate from TEPA parallels the formation of cyanuric acid from TEM described by Nadkarni *et al.* (1954):



II
TEM

III
Cyanuric acid

It is curious, however, that the majority of the TEPA escapes from the rat unchanged whereas TEM in mice at a comparable dose (2.0 mg./kg.) was found to be completely metabolized to cyanuric acid.

The separation of various tumour components after injection of labelled TEPA has not indicated localization of radioactive material in any particular cell fraction. Since the compound is partly metabolized into phosphate, the question arises whether the radioactivity fixed in the tissues represents combined drug. It is possible that localization of TEPA by combination with cell components is followed by breakdown of the complexes formed with the liberation of phosphate as illustrated above. The radioactive phosphate could then enter locally into the many cellular reactions involving phosphate, and account for the distribution found. The observed localization of radioactivity in the protein part of haemoglobin supports the view that the reaction of TEPA with cellular material is not necessarily followed by the loss of phosphate. More certain information of the site of reaction of the ethyleneimine group could be obtained by the use of a labelled atom in this three-membered ring.

Summary

1. Triethylenephosphoramide (TEPA) labelled with radioactive phosphorus has been prepared, its purity established chromatographically, and its anti-tumour activity confirmed.

2. Only a small part of the administered radioactivity was retained by the animal after 24 hr. No specific localization of radioactive material occurred in any rat tissue examined after doses which effectively inhibited the Walker carcinosarcoma and Jensen sarcoma. In tumour tissue the radioactivity was not associated with any particular cell component.

3. Over 80% of the injected dose was excreted in the urine in 24 hr. The majority of this was present as unchanged drug. The only metabolite detected was identified chromatographically as inorganic phosphate.

4. Although TEPA is a potent tumour inhibitor it does not appear to be very reactive chemically *in vivo*. The results suggest that its cytotoxic activity may be due to the interference by a very small proportion of the dose administered with some highly susceptible cellular mechanism.

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Brit. J. Pharmacol. (1955), **10**, 326.

SUPERFUSION AS A METHOD FOR THE STUDY OF DRUG ANTAGONISM

BY

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(RECEIVED MARCH 21, 1955)

In investigating constrictor substances in shed blood it became necessary to consider methods for the assay of 5-hydroxytryptamine (HT) in the presence of other pharmacologically active substances. Separation of some of these substances could be achieved by extraction with 95% acetone in which adenosine, substance P, and potassium salts are insoluble, whereas HT, histamine, acetylcholine, adrenaline, and noradrenaline are soluble (Amin, Crawford, and Gaddum, 1954). The problem thus became the determination of the amount of HT present in solutions containing histamine or ACh or both. Attempts were made to use the perfused rabbit ear (Holgate, 1949, 1953). With mepyramine 10^{-7} in the perfusate this preparation still showed a high degree of sensitivity to HT, but the method proved laborious for accurate assay. The rat uterus under conditions of superfusion showed great instability despite most careful prior preparation of the animal with stilboestrol. Very slight changes in experimental conditions gave rise to maximal contractions of the muscle invalidating all attempts at assay. The superfused rat colon gave good specificity for HT with high sensitivity, but with much variability between tissues obtained from different animals. The guinea-pig ileum was much more consistent in its responses, but contracted to HT, histamine, and ACh. A method involving the use of mepyramine and atropine was considered. This method had been used by Feldberg and Toh (1953) and by Dalgliesh, Toh, and Work (1953) in connexion with mixtures containing HT, histamine, and ACh together with substance P, the activity of the remaining substance P being determined after blocking HT with tryptamine (Gaddum, 1953). It was evident, however, there are conflicting opinions about the effect of atropine on the response of the ileum to HT. Some consider atropine to reduce markedly the response to HT in concentrations which also affect the response to ACh (Robertson, 1953; Rocha e Silva, Valle, and Picarelli, 1953), while others state that concentrations which abolish the effects of ACh and carbachol have but little effect upon the response to HT (Gaddum and Hameed, 1954; Gaddum, 1953). Similarly there is little information about the effect of mepyramine on the response of the ileum to HT, although Gaddum and Hameed (1954) state that it abolishes the effect of histamine in doses that have little or no effect on HT.

We were thus led to investigate the quantitative relationships of the antagonism by atropine and mepyramine of ACh, histamine, and HT. The technique of superfusion was used, since we had found this method both convenient and advantageous when studying the action of agonists (Cambridge and Holgate, 1954). The results obtained not only illustrate the limitations in the use of antagonists on the ileum for assay of HT in mixtures, but have yielded new information on the antagonism between atropine and HT.

METHODS

Superfusion Apparatus.—The apparatus used was modified from that described previously (Cambridge and Holgate, 1954), and is shown in diagrammatic form in Fig. 1. The tissue was suspended in air in a 15 cm. by 3 cm. water-jacketed chamber (K). This chamber was closed at the lower end by a perforated bung carrying a hook for tissue attachment, and, at the upper end, by a "Perspex" disc, slotted for the passage of a thread. A "Perspex" tube passing through the double wall of the chamber enabled a thermistor (S) to be placed below the tissue. Drugs and Tyrode solutions were contained in bottles (F and E) in the bath (G) and were driven from these by compressed air or oxygen respectively. These gases were fed in under pressure controlled by the water valves (BB) and recorded on manometers (CC), the actual entry to the bottles being determined by the opening of the electro-magnetic clamps (D). Passage of drugs and Tyrode from the bottles to the gut was through fine polyvinyl tubes contained in a small water jacket (H) to the water-jacketed glass warming

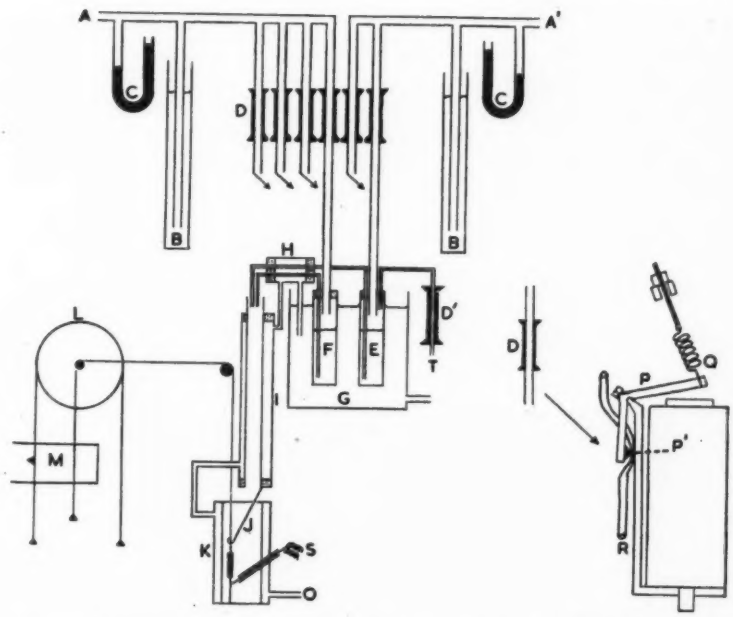

FIG. 1.—Diagram of superfusion apparatus. A, compressed air inlet, A', oxygen inlet; BB, water valves; CC, mercury manometers; D, electro-magnetic clamps controlling air and oxygen inlet to drug or superfusion fluid bottles; D', electro-magnetic clamp controlling pressure release; E, superfusion fluid bottles; F, drug bottles; G, warm water bath; H, small water jacket about outlet tubes; I, water-jacketed tube; J, glass finger; K, water-jacketed tissue chamber; L, pulley recording system; M, recording drum; O, inlet to water-jackets and bath; S, thermistor; T, pressure release tube. Inset: diagram of electro-magnetic clamp. P, armature with bar P'; Q, adjustable spring; R, tubing.

tube (I), and, finally, via a glass finger within the confines of the tissue chamber on to the upper thread attached to the tissue. Air and oxygen pressures were adjusted to give a final drop rate of 60/min., 20 drops being approximately equal to 1 ml. Viability of tissue for long periods was taken to indicate adequate oxygenation of superfusion fluid. Persistence of oxygen pressure in superfusion fluid bottles leading to "over-run" of fluid was eliminated by opening a pressure release clamp D' whenever D was closed. During the experiments the flow of superfusion fluid from bottles E was interrupted every 2½ to 3 min. for 40 sec., during which time 40 drops were applied to the gut from one of the drug bottles (F); the flow of Tyrode was then resumed. The flow of Tyrode actually ceased 3 to 5 sec. before the application of the drug. All operations were carried out automatically, using an apparatus developed from that described by Schild (1946, 1947). The time course of all events was further checked by direct observation.

The temperature in the bath (G) and the water-jackets was maintained at 30.0° C.

Recording System.—In previous experiments we used a frontal writing lever for recording gut movements, but in this series we have used a pulley system. The upper thread from the tissue, after leaving the chamber, passed over a "Perspex" pulley on pivot bearings, ran horizontally for 30 cm., was wound once round a further pulley 1.5 cm. in diameter to hang vertically and terminate in a counter-balance weight of 0.63 g. This second pulley was affixed to a large "Perspex" disc 15 cm. in diameter, drilled out for lightness and balance, the whole being mounted on pivot bearings. In a groove round the circumference of this disc ran a thread carrying a glass capillary writing point and, at its extremities, two counter-balance weights of approximately 1.0 g. to keep the thread taut. Both ends of this thread ran through slots in a horizontal brass bar which limited the excursion of the counter-balance weights so that the writing point did not pass beyond the upper and lower limits of the smoked drum. All grooves in the pulleys were of U-shaped cross-section to prevent binding of the threads. This system gave truly linear translation of gut contraction at a magnification of 10 : 1. A vibrator was bolted on to the main frame, this keeping the bearings free, and resulting in a high degree of sensitivity such that a weight of 0.1 g. applied to the thread attached to the gut gave a full excursion of the writing point.

Preparation of the Tissue.—In all experiments 3 to 4 cm. lengths of the terminal portion of the ileum of female virgin guinea-pigs weighing 150 to 250 g. were used, the pigs being starved 24 hr. before removal of the gut. After washing through with Tyrode, portions were tied with open ends. The use of animals within the weight range indicated gave a sensitivity to histamine similar to that found by Adam, Hardwick, and Spencer (1954), and higher than that previously reported by us.

Tyrode Solution.—The composition was (g./l.): NaCl 8.0, KCl 0.2, $CaCl_2$ 0.2, $MgCl_2$ 0.1, $NaHCO_3$ 1.0, NaH_2PO_4 0.05, and glucose 1.0. The *p*H lay between 8.0 and 8.4.

Drugs.—These were dissolved in Tyrode solution using the following salts: histamine acid phosphate, acetylcholine bromide, 5-hydroxytryptamine creatinine sulphate (kindly supplied by Dr. Merrell E. Speeter), atropine sulphate and mepyramine maleate. All concentrations referred to in this paper are in terms of ng./ml. of the base.

RESULTS

Antagonism of Histamine by Mepyramine

The antagonism between mepyramine and histamine lent itself best to preliminary investigation and will be described in detail as an example of the

methods used. Fig. 2 shows a typical example of the effect of one concentration of mepyramine on the response of the ileum to one concentration of histamine. The flow of superfusion fluid was interrupted every 2½ min. for the application (over a period of 40 sec.) of 2 ml. of Tyrode solution containing histamine (5 ng./ml.), thus producing the contractions shown at the beginning of the record. After at least 5 stable responses had been obtained antagonist solution was run over the tissue in place of Tyrode ; thus, during the period M, the superfusing fluid, but not the histamine solution, contained mepyramine 0.35 ng./ml. The amine, as in the period marked B. In the experiment illustrated, superfusion with mepyramine-free Tyrode was resumed after 40 min., but in other experiments it was found that the size of the response to histamine in this equilibrium state persisted unchanged although application of antagonist was continued for as long as 2 hr. Responses recorded during this period showed no greater variation than those recorded in the absence of mepyramine. The first one or two responses to histamine immediately following the change back to mepyramine-free Tyrode were often smaller than those during the equilibrium

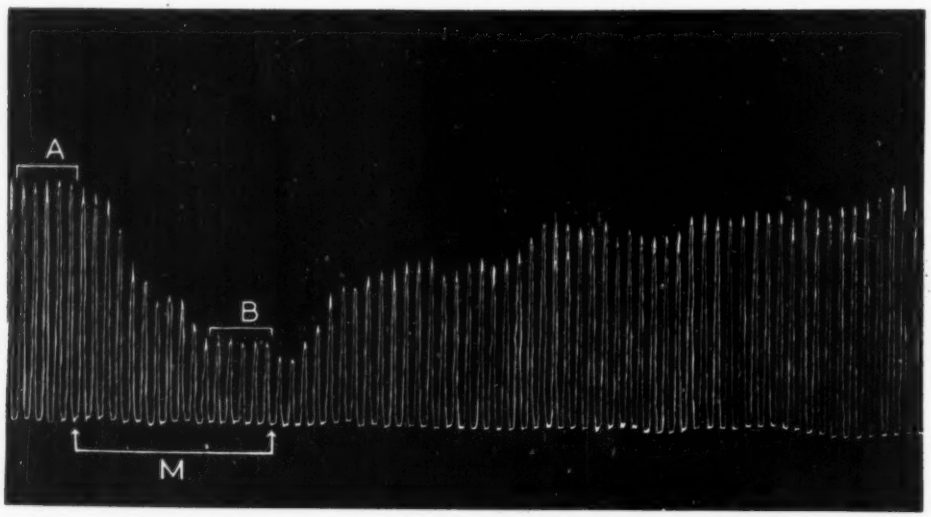

FIG. 2.—Typical record showing the depression of the response of the ileum to 5 ng./ml. histamine produced by 0.35 ng./ml. mepyramine in the superfusion fluid. Histamine application at 2½ min. intervals. Mepyramine applied during period indicated by M.

routine otherwise was unchanged and the decrease in the responses to histamine under these conditions is obvious. After replacement of the antagonist by mepyramine-free Tyrode the response to histamine increased gradually to its initial level.

The amount of drug given (40 drops) was well in excess of the minimum previously proved necessary for maximal accuracy (Cambridge and Holgate, 1954). At the beginning of every experiment a sensitizing concentration of agonist was chosen and repeatedly applied for 60 to 90 min. to ensure complete stability of response. Antagonists were not applied to the tissue until it had been under superfusion and drug stimulation for at least 2 hr.

Time Course of the Antagonism.—The effect of mepyramine applied in the way described increased, as in Fig. 2, over a period of 20 to 25 min. until the maximum reduction in the histamine response was obtained ; the effect then remained stable during the continued application of mepyr- state (Fig. 2), as was also observed by Schild (1947) using the bath technique. Thereafter the size of the contractions increased steadily, and after some 130 min. reached the initial size, at which point the tissue was ready for the application of another concentration of mepyramine.

Relationship Between Antagonism and Concentration of Mepyramine.—The results of observations such as those illustrated in Fig. 2 could be most conveniently expressed for many purposes in the following way. The average height of the contractions in the equilibrium period of antagonism (the mean of the 5 observations B) was expressed as a percentage of the initial height of contraction (the mean of the 5 observations A): in this example the effect of 0.35 ng./ml. of mepyramine was thus to reduce the response to histamine to 36%.

It was found that the effect expressed in this way did not vary appreciably in a large series of

tests using the same concentrations of both mepyramine and histamine on any one piece of gut, the sections being taken either from the same animal (within 10 cm. of the ileo-caecal junction) or from different pigs. When the concentration of mepyramine was varied, keeping that of histamine the same, the degree of antagonism produced by the mepyramine was in approximately linear relationship to the logarithm of the concentration—as in the example of Fig. 3, where 4 concentrations of mepyramine within a limited range were tested. Because of the constancy of behaviour of sections of gut from different guinea-pigs it was possible to use the same concentration of histamine in a large series of experiments. This concentration, and those of other agonists used, was chosen to give a response lying between 50 and 75% of the maximum possible contraction. In experiments where the effect of an antagonist on two agonists was investigated approximately equi-active concentrations of the agonists were used.

Some 22 experiments were performed in which different concentrations of mepyramine were tested on the contractions produced by 5 ng./ml. histamine, and the composite results of these experiments are shown in Fig. 4, line a. Scatter about the points has not been shown but was remarkably small, being of the order of $\pm 5\%$ except with very low concentrations of antagonist; in these circumstances it was frequently found that the mean response of the tissue to the agonist was uncertain

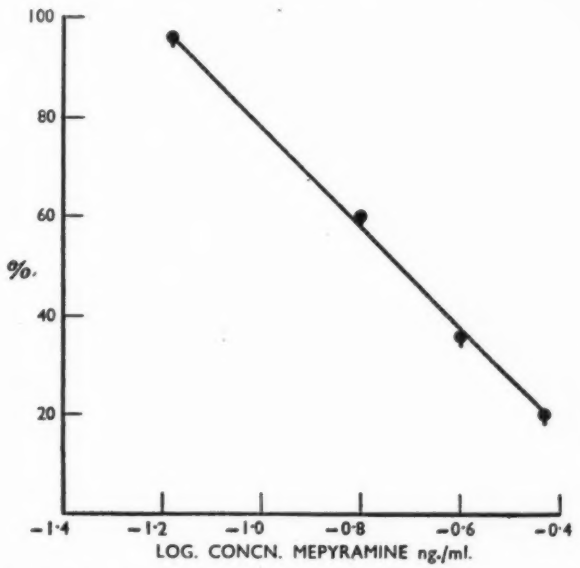

FIG. 3.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression of the response of the ileum to 5 ng./ml. histamine expressed as the percentage value described in text.

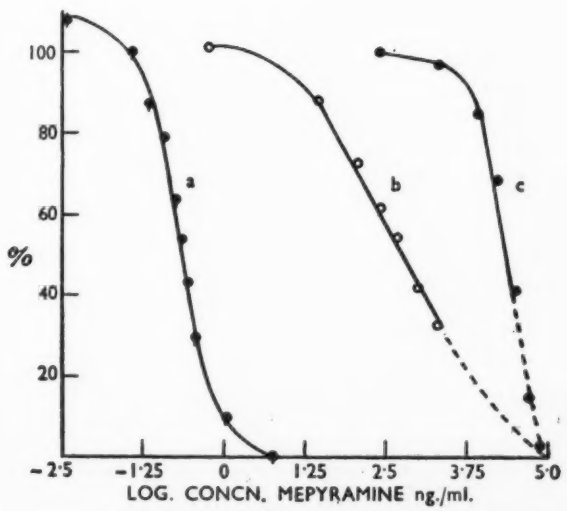

FIG. 4.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression in the response of the ileum to 5 ng./ml. histamine (a), 15 ng./ml. HT (b) and 5 ng./ml. acetylcholine (c), expressed as the percentage values described in text.

and was often slightly increased. Inconstancy of individual successive responses greater than that shown in the absence of inhibition was also a feature in the presence of low concentrations of antagonist.

Whereas in all preliminary experiments full recovery of response (as shown in Fig. 2) was obtained before application of a further concentration of antagonist, in later investigations into the same antagonist/agonist combination we found this to be unnecessary. In such investigations the first concentration applied was the lowest to be used and, after reaching equilibrium, a higher concentration was then applied, reducing the response to a new equilibrium state. By this means many more concentrations of antagonists could be tested within a limited time, the only assumption being that the sensitivity of the preparation to the agonist did not alter appreciably. That this assumption was valid was shown by the fact that curves obtained by this method did not differ from those obtained in the preliminary experiments.

In the foregoing experiments the same concentration of histamine was used throughout. Experiments were also performed in which contractions to 2 concentrations of histamine (in the example shown in Fig. 5—5.1 and 8.2 ng./ml.) were recorded alternately and the effect of different concentrations of mepyramine observed. In Fig. 5 the size of the mepyramine-reduced contractions (expressed as a percentage of the initial contraction to that concentration of histamine) is plotted

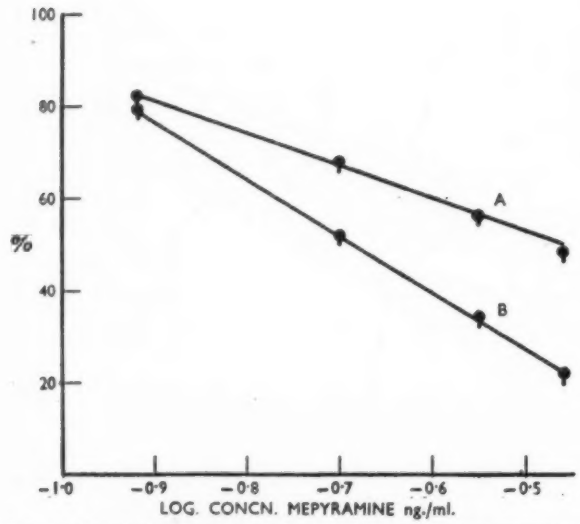

FIG. 5.—Graph showing the relationship between the log. concentration of mepyramine in the superfusion fluid and the depression of the response of the ileum to 8.2 (A) and 5.1 (B) ng./ml. histamine, the depression expressed as the percentage value described in the text.

against the log. concentration of mepyramine ; in all such experiments the lines were not parallel.

Dose/Response Curve of Histamine in the Presence of Mepyramine.—Fig. 6 shows the result of an experiment in which the log. concentration/response curve of histamine was determined before and during the equilibrium state of depression produced by 0.3 ng./ml. of mepyramine ; the two curves show no deviation from parallelism. The log. concentration/response curve plotted after recovery of the tissue from the action of mepyramine was not significantly different from the original, indicating the completeness of recovery under these experimental conditions.

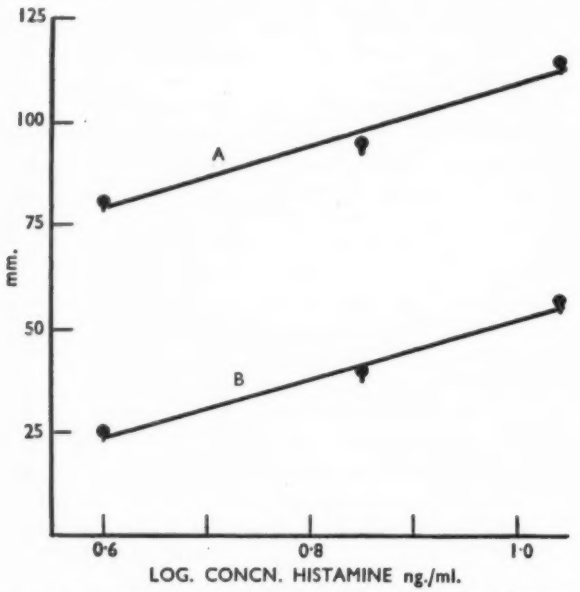

FIG. 6.—Log. concentration/response curves of histamine before (A) and during (B) the exposure of the ileum to mepyramine 0.3 ng./ml. in the superfusion fluid.

pA Values for Mepyramine Against Histamine.—Schild (1947) has defined the pA_x value. Accurate application of this over a wide range of dose is only possible if the log. concentration/response curve of the agonist in the presence of the antagonist is parallel to the curve in the absence of antagonist ; this has been shown above to be so for histamine and mepyramine.

We have determined *p*A values by the following procedure. Firstly, a log. concentration/response curve for histamine in the absence of mepyramine was constructed using doses of histamine of x, 0.5x, 0.2x, and 0.1x, the actual concentrations being chosen to lie on the linear part of the curve. From this curve it was found that the responses to 0.5x, 0.2x, and 0.1x were, in one example, 76%, 44%, and 20% of the contraction to x. Secondly, the effects of different concentrations of mepyramine were tested against responses produced by x ng./ml. of histamine (as a fixed concentration), so that a curve similar to that of Fig. 3 was plotted. From this line the concentrations of mepyramine necessary to reduce the response to x ng./ml. histamine to 76%, 44%, and 20% were 0.12 ng./ml., 0.24 ng./ml., and 0.39 ng./ml., and the negative logarithms to base 10 of the molar concentrations equivalent to these gave the pA_2, pA_5, and pA_{10} values in this test.

Schild (1947) and Reuse (1948) have measured *p*A values for histamine/mepyramine using the isolated gut in an organ bath. In their experiments the equilibrium state was not necessarily reached—that is, the size of the histamine contraction was still diminishing—so that it was necessary to define the time (2, 14, or 15 min.) after the introduction of the mepyramine at which the *p*A values were measured. In our experiments equilibrium was achieved and the values given in Table I might be designated pA_2E, pA_5E, or $pA_{10}E$ to

TABLE I

TABLE OF pA_xE VALUES FOR ATROPINE AND MEPYRAMINE AGAINST ACETYLCHOLINE, HT AND HISTAMINE

	Atropine			Mepyramine		
	ACh	HT	H	ACh	HT	H
pA_2	8·74	7·93	6·30	4·44	6·50	9·37
pA_5	8·37	6·00	5·95	4·14	6·04	9·08
pA_{10}	8·25	5·42	5·48	3·91	5·86	8·86

distinguish them from the pA_2 and pA_{10} (2 or 14 min.) values of Schild (1947) or the pA_2 and pA_{10} (15 min.) values of Reuse (1948). The values given in Table I do not differ greatly from those determined at the longer times of exposure by Schild and by Reuse.

Antagonism of Acetylcholine and 5-Hydroxytryptamine by Mepyramine

The action of mepyramine on the response of the ileum to ACh and to HT was studied by methods similar to the above. It was found advisable to make the applications of HT at intervals of 5 to 6 min. in order to avoid tachyphylaxis ; in practice these were alternated with applications of either ACh or histamine so that the depression curves for agonists could be determined simultaneously.

The time course of the antagonism of HT or ACh, using suitable concentrations of mepyramine, was similar to that of histamine ; thus, as before, the extent of the antagonism could be expressed as the percentage depression present at equilibrium. In Fig. 4 the effect of mepyramine against histamine (line a) is compared with its effect against HT (line b) in 11 experiments, and against ACh (line c) in 9 experiments. The interrupted sections of these lines indicate some degree of uncertainty as, at these high concentrations, mepyramine itself produced some contraction of the ileum. In these experiments the same concentration of agonist was given on every occasion, namely 15 ng./ml. for HT and 5 ng./ml. for ACh, these concentrations being approximately equal in activity to 5 ng./ml. of histamine.

Log. concentration/response curves for HT and ACh in the presence and absence of mepyramine were parallel in each instance. It was thus permissible to determine pA values by the procedure described above. These are given in Table I. The pA_2 and pA_{10} values for mepyramine/ACh are comparable with those reported by Schild (1947) ; the values for HT reflect its intermediate position between histamine and acetylcholine.

Antagonism of Acetylcholine, HT, and Histamine by Atropine

Acetylcholine.—Experiments as described for histamine/mepyramine using ACh and atropine gave very similar results. An equilibrium state was attained in which the depressed response persisted unchanged as long as atropine was continued ; there was a precise relationship between the degree of depression and the log. of the concentration of atropine used (Fig. 7, line a) ; log. concentration/response curves in the presence and absence of the antagonist were parallel, and

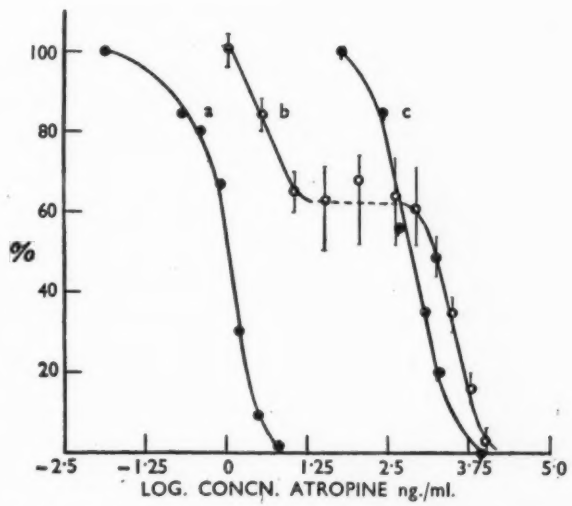

FIG. 7.—Graph showing the relationship between the log. concentration of atropine in the superfusion fluid and the depression of the response of the ileum to 10 ng./ml. acetylcholine (a), 20 ng./ml. HT (b) and 5 ng./ml. histamine (c) expressed as the percentage value described in the text.

depression/log. concentration of antagonist curves for 2 different concentrations of agonist were not parallel. The linear portion of the atropine/ACh curve (Fig. 7, line a) extends over a smaller range of atropine concentration and is somewhat steeper than the corresponding mepyramine/histamine curve (Fig. 4, line a). The lowest effective concentration of atropine is, at the same time, less than the corresponding mepyramine concentration, thus producing a final curve of somewhat different form. The pA values determined in the way described are given in Table I.

Owing to the period required for stabilization of response and for determining the dose/response relationship for one or more agonists, atropine was not applied until the tissue had been under superfusion for 2 to 3 hr. For this reason it was not established whether any change in sensitivity of the preparation to atropine occurred during the first 2 hr. following its removal from the animal, as reported by Flesch and Kun (1948). There was no change in sensitivity after this initial period as evidenced by the fact that the order in which the different concentrations of antagonist were applied made no difference to the final relationship obtained between the concentration of antagonist and the degree of depression of response to the agonist.

The equilibrium state was usually reached in 20 to 25 min. with mepyramine, and in 15 to 20 min. with atropine. Recovery from the effect of the antagonist was complete in 90 to 140 min. with

mepyramine and in 60 to 90 min. with atropine; presumably atropine diffused into and out of the tissue more rapidly than did mepyramine. Since in the above experiments the agonist solution did not contain antagonist there could have been some diffusion of the antagonist out of the tissue during the application of agonist. Addition of antagonist to agonist solutions in concentrations equal to those present in the superfusing fluid produced a slight reduction in the time necessary to reach the equilibrium state. With atropine, but not with mepyramine, this addition also produced a reduction in the response to the agonist greater than that produced when atropine was absent from the agonist solution. The difference, however, seemed too slight to warrant the technical inconvenience involved in changing the agonist solution every time a fresh concentration of antagonist was applied; this might, however, be necessary if any shorter acting antagonist were being tested.

Histamine.—As was expected, very high concentrations of atropine were necessary to produce reduction in the response to histamine. The form of the depression/log. concentration curve was similar to those previously determined and is shown in Fig. 7, line c; the *p*A values are given in Table I.

5-Hydroxytryptamine.—Experiments concerning the stability of the reduced response, and studies of log. concentration/response curves in the presence and absence of atropine, gave results similar to those for other agonist/antagonist combinations. However, the curve relating the depression of the response to HT to the log. of the concentration of the antagonist differs from the others (Fig. 7, line b). Six different sections of ileum were used. On each occasion depression of the response to HT by atropine began when the concentration of the latter reached approximately 1.0 ng./ml. With increasing concentration of atropine the depression increased until, when the concentration reached about 10 ng./ml., the response lay somewhere between 50 and 70% of the original. Increasing the concentration of antagonist over the range 10 to 1,000 ng./ml. produced no further depression of the response. Responses were, however, very variable in this range of atropine concentration. In those tissues where the response was 50% of the original in the presence of 10 ng./ml. of atropine, the response in the presence of concentrations up to 1,000 ng./ml. was also of the order of 50%. In tissues where the response had been reduced to 70% of the original by 10 ng./ml. of atropine this higher response was still seen although the concentration of antagonist was increased 100-fold. Increasing the concentration of atropine beyond 1.0 μg./ml. reduced the response to HT on a curve similar to that of the reduction of the response to histamine. The vertical limits on line b, Fig. 7, indicate the ranges in the 6 different tissues.

This characteristic behaviour of the response of the ileum to HT in the presence of different concentrations of atropine, as indicated by these results, has not previously been described. We feel that this should be taken into account in any consideration of the mode of action of HT upon the guinea-pig gut.

The "plateau" in the curve for depression described above is also indicated by the *p*A values (Table I). The pA_2 value lies near to that for atropine/ACh and the pA_{10} near to that for atropine/histamine. The pA_5 value, as it happens, lies near to that for atropine/histamine but, depending upon the exact position of the "plateau," might just as readily lie nearer to the pA_5 for atropine/ACh.

When using HT in concentrations above 20 ng./ml. some tachyphylaxis was observed unless applications were spaced at 6 min. intervals. In addition, when these higher concentrations of HT were used, it was frequently observed that they caused some interference with the response of the tissue to histamine. A second application of the same concentration of histamine following HT was always greater than the first, this occurring also in the presence of atropine. For this reason, as well as for that of tachyphylaxis, concentrations of HT above 20 ng./ml. were not used, and, moreover, concentrations at or below this figure were given alternately with ACh and not histamine.

Discussion

Use of the Ileum as an Assay Preparation for HT.—The development by Amin, Crawford, and Gaddum (1954) of a method for the extraction and separation of HT and substance P from tissues provides a means of overcoming the difficulty of assay of HT on the ileum in mixtures which might also contain substance P. The separation of the two substances by this method would appear to be almost complete, but the extract would be likely to contain, in addition to HT, histamine, ACh, adrenaline, and noradrenaline. If HT is to be assayed in the presence of histamine it can be seen from Fig. 4, lines a and b, that abolition of the effect of 5 ng./ml. of histamine can be achieved by a concentration of mepyramine which has practically no effect on the response of the ileum to

15 ng./ml. HT, hence the effect of HT could be satisfactorily separated from that of histamine in mixtures containing histamine and HT in the proportions of 1:3. But in rabbit's blood, for example, the proportion might be as high as 3:1 (Code, 1937 ; Holgate, 1953) and the concentration of mepyramine which would be necessary to abolish the activity of histamine under these conditions would very seriously reduce, if not abolish, the response of the ileum to the less active HT. Indeed, Cambridge and Holgate (1953) have shown that the remaining response in the presence of 10 ng./ml. mepyramine to a mixture of histamine and HT in the proportions of 1.5:1 was, in fact, due to histamine, the effect of HT having been completely abolished. Only if the content of histamine in the mixture were considerably less than that of HT would it be possible, using carefully chosen concentrations of mepyramine, to assay accurately the content of HT.

With regard to the assay of HT in the presence of ACh, from Fig. 7, lines a and b, it can be seen that the activity of 10 ng./ml. ACh would be abolished by a concentration of about 10 ng./ml. atropine, but the response to 20 ng./ml. HT would, in these circumstances, be reduced to about 60 to 70% of the original. In addition this response would be brought into the range of greatest variability.

Clearly, even without the complications due to the possible presence of adrenaline and noradrenaline, the guinea-pig ileum in the presence of atropine and mepyramine is a most unsuitable preparation for the assay of HT in mixtures containing ACh and histamine—unless the proportion of the activity due to HT is very high ; even in this event the concentrations of the antagonists used must be chosen with care.

Methods for Expressing the Activity of Antagonists.—Gaddum (1926), Winder, Kaiser, Anderson, and Glassco (1946), Miller, Becker, and Tainter (1948), and Rapport and Koelle (1953) all used a fixed concentration of agonist and compared the effect of various antagonists by measuring the reduction they produced in the response of the tissue. For example, Winder *et al.* (1946) expressed the power of the antagonist as that concentration required to produce 70% depression in the response (the ED70 value), the concentration then being expressed in terms of diphenhydramine equivalents. Miller *et al.* (1948) devised the ED50 value—the concentration of spasmolytic agent capable of producing a 75% reduction of a maximal spasm in one half of the test preparations. The concentration of antagonist was expressed as the log. of its dilution, the *p*D value. Figs. 3, 4, 5, and 7 give results of experiments of this type ; the ED70 value, for example, could be calculated from them. The choice of concentration of agonist has been made by different authors in different ways. Winder *et al.* (1946) speak of a " pre-determined standard spasm " and Miller *et al.* (1948) of a " maximal spasm." We chose a concentration which produced a response 50 to 75% of the maximal contraction possible in a 3 to 4 cm. length of ileum pulling against a load of 0.2 to 0.3 g. If a higher concentration of agonist was used (Fig. 5, line A) the same degree of antagonism could only be produced by using a higher concentration of antagonist, which would lead to a higher ED70 value. In the early phases of our investigations this fact led us to the use of what we termed the " R " value, which is the ratio between the concentration of the agonist and that of the antagonist required to produce a constant amount of reduction in the response. This value has more recently been better named the " drug ratio " by Gaddum, Hameed, Hathway, and Stephens (1955) and has been fully discussed by these authors. We noticed that this value was remarkably constant over a relatively wide range of concentration of stimulant drug and realized that it would form a useful way of comparing rapidly the activity of many antagonists against one agonist. All these expressions of activity are limited in their application and serve mainly in the comparison of potencies of different antagonists.

Some authors allowed the antagonist to act for arbitrarily chosen periods of time ; thus, Winder *et al.* (1946) introduced the antagonist into the bath 1 min. before the agonist, a time which they regarded as suitable since the results obtained then represented " a combination of speed of penetration to the site of action with the intrinsic intensity of prophylactic action." Miller *et al.* (1948) measured the reduction of the maximal spasm after the antagonist had been present in the bath for 2 min. Rapport and Koelle (1953) measured the percentage reduction of the response produced by equi-active doses of ACh, HT, and histamine after exposure to known concentrations of the antagonists for 5 min., adding the agonist to the bath while the antagonist was still present. In some experiments the agonists were tested at 1 min. intervals in the presence of each other, while in other experiments the bath was washed out with the antagonist between each addition. Thus the period of contact with the antagonist increased during the test, and the authors comment on the significance of the duration of exposure to antago-

nists in such experiments. Owing to the cumulative and persistent action of atropine under these conditions, Rapport and Koelle were unable to demonstrate the gradual development of block with increasing concentration. It is clear from Fig. 2 that the duration of exposure will greatly influence the concentration of antagonist necessary to produce a given degree of depression, a fact clearly stated by Gaddum (1926) and by Schild (1947). The method of superfusion has the clear advantage over other methods in that the time course of antagonism can be followed and the equilibrium state can be appreciated.

The pattern of recovery of response, as illustrated in Fig. 2, has been used as a means of studying antagonists. For example, Rocha e Silva and Beraldo (1948), and Beraldo and Rocha e Silva (1949), investigated this aspect of the response to a standard dose of an agonist after exposure of the tissue to an antagonist for 1 min., the time for 50% recovery being the R50 value. From this type of experiment Rocha e Silva, Valle, and Picarelli (1953) concluded that the atropine antagonism of HT was similar to the antagonism of ACh by atropine because of the similarity of the recovery curves. From our experience the recovery curve seems to be a property of the antagonist itself rather than a reflection of the mode of action of the agonist.

The *p*A value of Schild (1947) is the most satisfactory term by which to express antagonism since it is independent of the concentration of agonist—provided this is submaximal and that the log. concentration/response curves in the presence and absence of the antagonist are parallel (Schild, 1949). Our results are *p*A values at the equilibrium state, which can be readily achieved with superfused tissues; the necessity of arbitrarily choosing the time of exposure to the antagonist is thus avoided.

Antagonism of 5-Hydroxytryptamine by Atropine.—Expressing the antagonism of HT by atropine, both in terms of the *p*A values and by the curve relating the depression of response to the concentration of antagonist, has revealed certain characteristic features. Depression of the response of the ileum to HT to about 60% of the original can be achieved by low concentrations of atropine, similar to those affecting equi-active concentrations of ACh. For complete abolition of response much higher concentrations of atropine are necessary, similar to those required for the abolition of the response to equi-active concentrations of histamine. These findings may reconcile the conflicting views of previous authors regarding this antagonism.

Summary

1. A method is described for the study of drug antagonism using the superfusion technique.

2. This method can be adapted to any of the standard means of expressing antagonism; it has the added advantages over the bath technique of (*a*) exact determination of equilibrium conditions, (*b*) improved viability of the tissue, and (*c*) the possibility of determining the effects of many concentrations of one or more antagonists on the same preparation.

3. In applying this method to the study of the antagonism of HT by atropine, the curve relating the degree of depression of response to the concentration of atropine has been shown to have a characteristic form not previously described. This must clearly be taken into account in any discussion concerning the mode of action of HT upon the isolated guinea-pig ileum.

4. The method has been used to investigate the value of the atropine- and mepyramine-treated guinea-pig ileum as an accurate assay preparation for the determination of HT in mixtures containing ACh and histamine. It is concluded that this preparation is unsuitable except for mixtures containing relatively large amounts of HT.

5. Means of expressing the activity of antagonists are discussed. It is concluded that the curve relating the degree of depression of the agonistic response to the concentration of the antagonist, taken in conjunction with the *p*A value at equilibrium, affords the most comparable way of expressing the activity of one or more antagonists against a group of agonists. The drug ratio, on the other hand, is more useful when comparing the activities of a number of antagonists upon one agonist.

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THE EFFECT OF CHLORPROMAZINE ON ADRENOCORTICAL ACTIVITY IN STRESS

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(RECEIVED APRIL 2, 1955)

Avon, Chambon, and Voisin (1953) claimed that doses of 10 to 50 mg./kg. of chlorpromazine blocked the effects of surgical stress and of formalin injections on the rat's adrenal ascorbic acid. Holzbauer and Vogt (1954), on the other hand, found that the release of ACTH caused by surgical stress or by injection of adrenaline was not blocked by doses of 10 mg./kg. of chlorpromazine. They also showed that this dose of chlorpromazine alone caused some release of ACTH. For this reason they allowed 3 hr. to elapse between injecting the chlorpromazine and applying the stress. Accordingly, it seemed to be of interest to determine whether or not smaller doses of chlorpromazine, which failed to release ACTH, would block the effects of stress on the adrenal cortex, since a shorter time between giving the drug and applying the stress could then be used without obscuring the issue.

METHODS

Animals.—Litter-mate buck rats of the Wistar strain were used. They were kept on a diet of cubes, cabbage, and water in a room thermostatically controlled at 70° F. Neither food nor water was withheld at any time during the experiments.

Drugs.—All drug solutions were freshly made in normal saline and were injected subcutaneously unless otherwise stated. The doses of histamine are in terms of the hydrochloride, and those of adrenaline in terms of the *laevo*-rotatory base.

Surgical Stress.—The animals were anaesthetized with ether, and a mock adrenalectomy was performed through a midline incision in the skin of the back. The wound was sutured and the animals were allowed to recover.

Demedullation of Adrenal Glands.—Adrenal glands were demedullated by the technique of Evans (1936). The demedullated rats were used between 75 and 90 days after operation.

Extraction of Adrenal Glands.—All animals were killed by a blow on the back of the head 1½ hr. after applying the stress. The adrenals were removed, weighed, and extracted separately as described by Nasmyth (1954). The ascorbic acid content of the extracts was estimated by the technique of Roe and Kuether (1943).

Record of Rat's Blood Pressure.—Animals were anaesthetized with 7 ml./kg. of 25% urethane solution given subcutaneously. Pressure was recorded from the carotid artery using the manometer described by Condon (1951). Heparin was introduced into the arterial cannula to prevent clotting. Doses of histamine were injected into the cannulated jugular vein; chlorpromazine and mepyramine maleate were given subcutaneously on the chest.

RESULTS

Chlorpromazine Effect.—Within a few minutes of the subcutaneous injection of 10 mg./kg. of chlorpromazine, the animals became lethargic. One and a half hours after the injection the adrenal ascorbic acid content had fallen to 68.4% of its normal level. When 2.5 mg./kg. of chlorpromazine was injected, followed by 1 ml./kg. of normal saline 30 min. later, the same signs of lethargy were seen. One and a half hours after the dose of saline the rats were killed; their ascorbic acid content was not significantly different from that of rats given saline alone. These observations (Table I) established the fact that the higher dose of chlorpromazine caused a release of ACTH, which was evident 1½ hr. after injection, whereas the lower dose did not. Accordingly, a dose of 2.5 mg./kg. of chlorpromazine was used to assess the effect of the drug on the release of ACTH caused by various forms of stress.

Adrenaline Effect.—Two doses of adrenaline were used, which were calculated to produce submaximal effects on the adrenal ascorbic acid. Injection of 50 μg./kg. of adrenaline caused a fall in adrenal ascorbic acid to 62.7% of the control after 1½ hr. The smaller dose of 25 μg./kg. caused a fall of 91% which was significant ($P<0.05$). When the animals were given chlorpromazine 30 min. before these doses of adrenaline, bigger falls in the

TABLE I
THE EFFECT OF VARIOUS STRESSES ON THE ASCORBIC ACID CONTENT OF RATS' ADRENAL GLANDS

	Chlorpromazine or Mepyramine 2·5 mg./kg. 30 min. before Stress	Stress	Duration of Stress (hr.)	Ascorbic Acid Content mg./100 g. Gland ± S.E.	Ascorbic Acid as % Normal	No. of Rats
Normal ..	Nil	1 ml./kg. 0·9% NaCl	1½	425 ± 9·3	100	18
	,,	10 mg./kg. chlorpromazine	1½	291 ± 29·0	68·4	3
	,,	10 ,, histamine	1½	276 ± 17·3	64·9	3
	,,	100 ,, ,,	1½	199 ± 3·9	46·8	4
	,,	50 μg./kg. adrenaline	1½	267 ± 6·9	62·7	3
	,,	25 ,, ,,	1½	387 ± 16·1	91·0	3
	,,	Surgical	1½	175 ± 16·4	41·2	4
	Chlorpromazine	1 ml./kg. 0·9% NaCl	1½	427 ± 8·2	100	16
	,,	10 mg./kg. histamine	1½	372 ± 24·1	87·5	6
	,,	100 ,, ,,	1½	210 ± 10·6	49·3	4
	,,	50 μg./kg. adrenaline	1½	213 ± 6·6	50·2	3
	,,	25 ,, ,,	1½	337 ± 19·7	79·3	3
	,,	Surgical	1½	168 ± 8·0	39·5	5
	Nil	2·5 mg./kg. mepyramine	1½	342 ± 8·8	80·6	3
	Mepyramine	10 ,, histamine	1½	341 ± 7·6	80·3	3
Demedullated	Nil	1 ml./kg. 0·9% NaCl	1½	298 ± 9·7	100	7
	,,	100 mg./kg. histamine	1½	152 ± 2·5	51	6
	,,	Surgical	1½	222 ± 6·3	74·5	4
	Chlorpromazine	1 ml./kg. 0·9% NaCl	1½	313 ± 7·6	100	4
	,,	100 mg./kg. histamine	1½	212 ± 5·7	67·7	5
	,,	Surgical	1½	218 ± 18·0	73·5	3

adrenal ascorbic acid were recorded (Table I). Only the difference between the falls due to the larger dose when given alone and when given after chlorpromazine was significant ($P<0.01$). These observations showed that chlorpromazine reinforced the release of ACTH caused by small doses of adrenaline.

Histamine Effect.—The subcutaneous injection of 10 mg./kg. of histamine produced no obvious signs, but 1½ hr. after the dose the adrenal ascorbic acid had fallen to 64.9% of the control. Ten times the dose of histamine caused prostration and a fall in the adrenal ascorbic acid to 46.8% of the control. When chlorpromazine was injected 30 min. before the histamine, the release of ACTH caused by the smaller dose was inhibited ($P<0.01$), but that caused by the larger dose was not significantly affected.

Since chlorpromazine did not block the release of ACTH caused by the smallest dose of adrenaline, the difference between its effects on large and small doses of histamine could be explained by supposing that the large dose, but not the small one, caused a substantial release of adrenaline from the adrenal medulla. The experiments with the large dose of histamine were therefore repeated in rats with demedullated adrenal glands.

One and a half hours after a dose of 100 mg./kg. of histamine in these animals, the ascorbic acid level of the adrenal glands had fallen to 51% of the control value. When the demedullated rats were given chlorpromazine before the dose of histamine, the fall in the adrenal ascorbic acid was inhibited. The difference between the two groups was significant ($P<0.01$). The results are given in Table I.

Surgical Stress.—Mock adrenalectomy under ether anaesthesia caused a profound fall in the adrenal ascorbic acid. One and a half hours after completion of the operation the glands contained only 41.2% of the control amount. The injection of chlorpromazine, 30 min. before performing the operation, did not alter the adrenal response (Table I).

As the operation was performed under ether, there was probably a considerable release of adrenaline from the adrenal medulla. For this reason the experiment was repeated in rats with demedullated adrenal glands. In these animals the response of the adrenal ascorbic acid was less than in normal animals, and it was unaffected by the prior injection of chlorpromazine. Without chlorpromazine the ascorbic acid level fell to 74.5% of the control 1½ hr. after the operation; with chlorpromazine it fell to 73.5%. These results (Table I) indicate that the release of medullary adrenaline caused by this procedure contributes to the release of ACTH from the pituitary gland. Furthermore, response to the stress which remains in the absence of medullary secretion is unaffected by chlorpromazine.

The Effect of Mepyramine Maleate on Histamine Stress.—It seemed possible that the ability of chlorpromazine to inhibit the release of ACTH caused by small doses of histamine in normal rats, and that caused by large doses in demedullated rats,

might be due to its antihistamine activity. The effect of a similar dose of mepyramine maleate given 30 min. before the small dose of histamine in normal rats was therefore determined. The results shown in Table I indicate clearly that mepyramine is effective in inhibiting the release of ACTH caused by small doses of histamine, though the mepyramine itself seems to have produced some fall in the adrenal ascorbic acid. The fact that 2.5 mg./kg. of mepyramine maleate, given subcutaneously, causes some fall in the adrenal ascorbic acid obscures to some extent the degree to which it is capable of inhibiting the release of ACTH by histamine, since two stresses are not simply additive.

Comparison of the Antihistaminic Activities of Chlorpromazine and Mepyramine on the Rat's Blood Pressure.—Since it was suspected that the effect of chlorpromazine was due to its activity as an antihistamine, it was of some interest to make an assessment of this property in the rat. The rat is well known to be insensitive to histamine, but the blood pressure usually gives fairly constant responses to repeated doses, and the assessment was accordingly made using this phenomenon. The procedure adopted was to find the smallest dose of histamine which would just cause a fall in blood pressure, then to re-determine the dose 30 min. after the subcutaneous injection of 2.5 mg./kg. of either mepyramine or chlorpromazine. In eight such experiments the average ratio of antihistamine activity of mepyramine and chlorpromazine was 7:1 respectively.

Discussion

It has been shown that chlorpromazine does not reduce the release of ACTH in surgical stress or in the stress caused by small doses of adrenaline, which confirms the observation of Holzbauer and Vogt (1954). Chlorpromazine did, however, inhibit the response of the adrenal ascorbic acid to small doses of histamine, but the response to large doses of histamine was only inhibited in animals with demedullated adrenal glands. The response to a large dose of histamine or to surgical stress was less in adrenal-demedullated than in normal animals, suggesting that medullary adrenaline normally contributes to the release of ACTH in both these forms of stress. The effect in adrenal-demedullated animals could be inhibited by chlorpromazine if the stressing agent was histamine, but not if it was trauma. Since the possibility of secretion from the adrenal medulla had been eliminated in these animals, it is probable that the observed response of the adrenal ascorbic acid in each type of stress was mediated by the hypothalamus. The action of chlorpromazine in inhibiting the effect of large doses of histamine in adrenal-demedullated rats could not, therefore, be attributed to any effect that it may have on the hypothalamus, since any action in that site must also have affected the response to surgical stress.

The question then arises, by what mechanism does chlorpromazine inhibit the release of ACTH by histamine ? It seems most likely to do so by blocking the peripheral effects of histamine. This view is supported by the experiments on the response of the rat's blood pressure to histamine before and after doses of chlorpromazine and mepyramine, which indicate that chlorpromazine has a considerable antihistamine effect. Furthermore, the more potent antihistamine, mepyramine maleate, was shown to produce a more complete inhibition of the release of ACTH by histamine—which is in accordance with the results of Halpern and Benos (1952).

The observation that chlorpromazine significantly increased the fall in adrenal ascorbic acid caused by 50 μg./kg. adrenaline is interesting. The mean fall caused by 25 μg./kg. of adrenaline was similarly increased, but not significantly, since the standard error of the mean was large and reflected the fact that this dose was uncertain in its effect on the pituitary. Such an increase in the effect of adrenaline on the adrenal ascorbic acid was not observed by Vogt and Holzbauer. However, they used 200 μg./kg. of adrenaline, which would produce a nearly maximal effect. The explanation of the phenomenon is not obvious, but may simply be an example of synergism.

Summary

1. The subcutaneous injection of 10 mg./kg. of chlorpromazine to rats reduced the adrenal ascorbic acid. No reduction was seen when a dose of 2.5 mg./kg. was given.

2. The injection of 2.5 mg./kg. of chlorpromazine did not block the release of ACTH caused by adrenaline, by surgical stress, or by large doses of histamine in the normal rat. The effect of a small dose of histamine was inhibited.

3. The release of ACTH by large doses of histamine and by surgical stress was reduced in demedullated rats. The remaining release of ACTH in these animals was inhibited by chlorpromazine when the stressing agent was histamine, but not when it was trauma.

4. Chlorpromazine was shown to be 7 times less active than mepyramine in blocking the effects of histamine on the rat's blood pressure.

5. The subcutaneous injection of 2.5 mg./kg. of mepyramine maleate caused a small fall in the adrenal ascorbic acid. Histamine, 10 mg./kg., injected subsequently, did not add to the fall caused by the mepyramine alone.

6. It is concluded that the role of the hypothalamus and that of medullary adrenaline in the release of ACTH is not directly affected by chlorpromazine. Chlorpromazine blocks the action of histamine by a peripheral antihistamine effect.

My thanks are due to Dr. H. C. Stewart for his interest and encouragement, and to Miss B. Morris for technical assistance. I am grateful to Messrs. May & Baker, Ltd., for a generous supply of chlorpromazine, and to the Sir Halley Stewart Trust for continued financial support.

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CHEMICAL CONSTITUTION AND ANTHELMINTHIC ACTIVITY OF CYCLIC ANALOGUES OF PHENOTHIAZINE

BY

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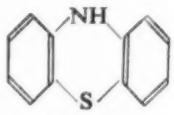
(RECEIVED APRIL 18, 1955)

Thiodiphenylamine, or phenothiazine (I), is used extensively as an anthelminthic, particularly for the treatment of nematode infestations of the alimentary tract of sheep (Harwood, 1953). This drug is of particular interest because, compared to many other anthelminthics, it is relatively specific in its action, and because it forms a variety of oxidation products in treated animals (Lipson, 1940; De Eds and Thomas, 1942; Clare, 1947; Clare, Whitten, and Filmer, 1947; Harpur, Swales, and Denstedt, 1950). It is probable that phenothiazine itself, rather than its oxidation products, is the effective anthelminthic (Esserman, 1952). On the other hand, the oxidation products inhibit a number of mammalian enzyme systems (Collier, 1940; Collier and Allen, 1942a, 1942b; Collier and Allenby, 1952), and many of the toxic signs in treated animals are probably due to these compounds (Collier and Allen, 1942a; Clare *et al.*, 1947; Whitten, 1948). We decided to examine the



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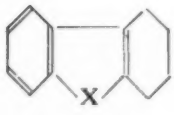

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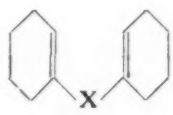

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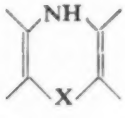


V

anthelminthic activity of a number of cyclic analogues of phenothiazine, comprising the various possible combinations of X and Y in structure II (where X or Y or both may be carbon, nitrogen, oxygen, sulphur or selenium) and structure III (where X may be carbon, nitrogen, oxygen, or sulphur). Structures II and III represent tricyclic isosteres of phenothiazine, whereas structure IV—where X is nitrogen or sulphur—represents the corresponding open-chain analogues.

In this paper the anthelminthic activity of 25 analogues of phenothiazine of type II, III, and IV is reported.

MATERIALS AND METHODS

Preparation of Compounds.—All compounds were prepared by published methods. They were of analytical purity and had the melting points recorded in the literature.

Anthelminthic Activity.—Natural infestations of mixed populations of *Syphacia obvelata* and *Aspiculuris tetraptera* were used for assessing anthelminthic activity. There was no obvious difference in the reactions of these two parasites to phenothiazine, and, as a rule, no attempt was made to separate them when counting the parasites in the host or in the faeces.

The compounds were administered by a stomach tube to mice lightly anaesthetized with ether. Each mouse received the drug suspended in 0.4 ml. of 1/500 (v/v) "Wetsit," an anionic detergent. "Wetsit" alone showed no activity. The faeces of each mouse were collected separately on damp filter paper for 24 hr., after which the mice were killed. The parasites in the faeces of each mouse, and those in the colon, caecum, and rectum were counted. Activity was assessed as $\frac{\text{parasites in faeces}}{\text{total parasites found}} \times 100$.

When *post mortem* examination showed that the compound was retained in the stomach of the treated animal for long periods (as with phenoselenazine), tests were extended to cover 48 hr. In order to determine the action of phenothiazine at different time intervals, 7 mice were given 1 g./kg., the faeces collected 4, 19, 24, 27, and 44 hr. after the dose, and the activity at the different times assessed.

The mice were not fasted before or during the experiments. At least 5 mice were used in each test and the total number of parasites/mouse was usually well in excess of 80.

Results

Phenothiazine.—The results of an experiment with phenothiazine are shown in Fig. 1. The linear regression is significant ($P<0.001$).

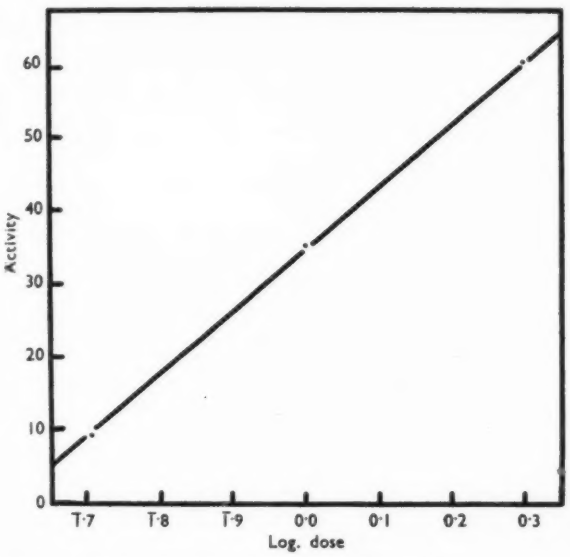


FIG. 1.—The relation between log. dose (g./kg.) of phenothiazine and activity. Activity was assessed as $\frac{\text{parasites in faeces}}{\text{total parasites}} \times 100$. The linear regression was significant ($P < 0.001$).

Fig. 2 shows that the maximum effect of a dose of 1 g./kg. of phenothiazine was attained in 24 hr. The graph shows the results from 6 of the

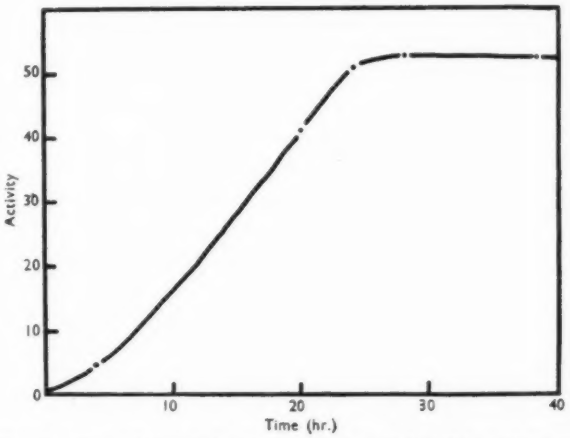
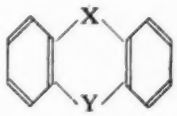


FIG. 2.—The relation between time and anthelminthic activity of phenothiazine (1·0 g./kg.). Activity as in Fig. 1. The standard deviations as percentages of the means were: 48 (0–4 hr.), 75 (0–19 hr.), 59 (0–24 hr.), and 55 (0–38 hr.).

7 mice treated. The remaining mouse became moribund after treatment and did not eat; worms continued to appear in the faeces for 44 hr. This result was quite different from all the others, and it was considered that the discrepancy was due to the moribund condition of the animal. From this and other experiments, it appeared that results obtained with animals which were markedly affected by the treatment were misleading, and they were therefore neglected.

Cyclic Analogues of Phenothiazine.—The results obtained with the active members of the series are summarized in Table I. Statistical analysis showed

TABLE I
ACTIVE TRICYCLIC PHENOTHIAZINE ANALOGUES OF STRUCTURE II



Compound	X	Y	Activity at 1 g./kg.	Activity at 2 g./kg.
Phenothiazine	NH	S	38	80
Phenoxazine	NH	O	—	80
Phenothiazine S-oxide	NH	SO	16	—
Acridine	N	CH	12	—
Thianthren	S	S	10	—
Dibenzdioxin	O	O	—	20

that there was no significant difference between the activities of phenothiazine and phenoxazine, and that these substances were significantly more active than the other compounds tested.

No activity was observed with phenoselenazine (II; X=NH, Y=Se), phenothiazine S-dioxide (X=NH, Y=SO_2), dihydroacridine (X=NH, Y=CH), acridone (X=NH, Y=CO), phenazine (X=N, Y=N), phenoxthiin (X=O, Y=S), anthracene (X=CH, Y=CH), xanthone (X=CO, Y=O), or thioxanthone (X=CO, Y=S).

No activity was observed with any of the compounds with structure III that were tested: carbazole (X=NH), dibenzfuran (X=O), dibenzthiophen (X=S), dibenzthiophen S-oxide (X=SO), or dioxide (X=SO_2), fluorenone (X=CO), fluorene (X=CH_2) or phenanthrene (X=CH:CH).

Two compounds of the structure IV were examined. Diphenyl sulphide (X=S) was inactive, and diphenylamine (X=NH) showed only slight activity at a dose of 2 g./kg.

Discussion

The number of tests on each compound was sufficient only to indicate their approximate relative activities. However, for this part of the investigation it was required only to separate compounds of high activity from those of low

activity, and the minimum number of tests for this purpose was carried out. It appears possible that the optimal requirements for anthelminthic activity may be the presence of the central ring-system (V) containing an -NH group and a hetero-atom X (such as oxygen or sulphur) possessing one or more "lone pairs" of electrons. Further work on this topic is in progress.

SUMMARY

1. An examination of the anthelminthic activity of 23 tricyclic and 2 acyclic analogues of phenothiazine showed that only phenothiazine and phenoxazine possessed appreciable activity when tested against mixed infestations of *Syphacia obvelata* and *Aspiculuris tetraptera*.

2. The significance of the results is discussed.

The authors are indebted to Mr. R. Simmonds for technical assistance and to Mr. V. Bofinger, of C.S.I.R.O. Division of Mathematical Statistics, for advice. One of us (G. P. W.) wishes to thank the Division of Animal Health and Production for the award of an Ian McMaster Scholarship. A grant from the Agricultural Research Council for technical assistance is also acknowledged.

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Brit. J. Pharmacol. (1955), **10**, 343.

ANTIFUNGAL ACTIVITIES OF BIS*ISO*QUINOLINIUM AND BISQUINOLINIUM SALTS

BY

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(RECEIVED APRIL 27, 1955)

We have previously reported (Collier, Potter, and Taylor, 1953) that certain polymethylene bis*iso*quinolinium salts powerfully inhibit bacterial growth *in vitro*. In spite of the relatively high subcutaneous toxicity of these compounds in mice it seemed possible that they might safely be applied to skin. They were therefore examined against fungi which infect keratinous structures of man and animals for inhibitory activity, which some of them proved to possess in high degree. Consequently further members of the bis*iso*quinolinium series and some corresponding bisquinolinium salts were prepared and their antifungal and toxic properties explored.

METHODS

Compounds.—The compounds are described in detail in the chemical section. In the biological section they are described by abbreviations corresponding to those adopted by Barlow and Ing (1948). Thus tetradecamethylene bis*iso*quinolinium is abbreviated to BIQ 14 and the corresponding quinolinium to BQ 14. Generally the quaternary iodides were used; but to obtain more concentrated solutions the methosulphates of BIQ 14 and BIQ 16 were employed.

Antifungal Tests.—In vitro antifungal properties were investigated in 11 strains of the 6 species named in Table I according to the terminology adopted by Conant, Smith, Baker, Callaway, and Martin (1954). Seven of the strains used had been isolated from man and the remainder from domestic animals. In all experiments, except those comparing the sensitiveness of different strains or species to drugs, we used a single strain of *Trichophyton mentagrophytes* that had been isolated from man.

Fungistatic Activity.—Tests of growth inhibition were carried out in Sabouraud's broth, composed of 1% peptone ("Eupeptone" No. 2, A. & H.) and 4% glucose in water. Drugs were serially diluted by 1 in 2 in 2 ml. volumes of broth and autoclaved at 10 lb./sq. in. for 10 min. The dilutions were inoculated with a ground suspension of fungal culture in Sabouraud's broth. After inoculation, tubes were incubated at 27° C. and read by eye after 3 days and subsequently. End-points were expressed as the minimal inhibitory concentration (MIC) and final results as the geometric mean MIC of several figures obtained independently.

Several materials were examined for ability to antagonize the growth inhibition exerted by BIQ 14 or BIQ 16. In addition to those listed in Table II, bovine hair and serum, dog hair and rabbit serum were examined. The materials were included in the media before autoclaving, except sera and lecithins, which were added aseptically to sterilized media.

Fungicidal Activity.—In tests of fungicidal activity a suspension containing about 5,000,000 spores/ml. saline was prepared from a 7–10 day culture of *T. mentagrophytes*. Tubes containing 2 ml. volumes of serially diluted drug were inoculated with 0.04 ml. spore suspension and incubated. After a measured time each tube was then shaken and one loopful (4 mm. diameter) transferred into 4 ml. 2% dried bovine bile ("Bacto-Oxgall") in Sabouraud's broth. The bile inhibited the antifungal action of any drug transferred with the inoculum. These subcultures were examined for fungal growth after 14 days' incubation at 27° C. Two series of fungicidal tests were carried out. In one, spore suspensions were incubated in concentrated solutions of drug in saline for periods between 10 min. and 24 hr. at 20° C., phenol being used for reference. In the other series, spore suspensions were incubated in drug solutions in Sabouraud's broth at 27° C. for up to 7 days.

Toxicity Tests.—In acute subcutaneous and oral toxicity tests in mice, drugs were administered in aqueous solution. The method of Miller and Tainter (1944) was used to estimate LD50 and its standard error. Tests of intradermal toxicity in rabbits were carried out by injecting 0.04 ml. of solutions of drug in saline into the shaved skin. In all acute toxicity tests the effects of drugs were observed for 7 days after treatment.

In subacute toxicity tests in rabbits, drugs were administered once daily, except on Saturdays and

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Sundays. Saline solutions of drugs were instilled into one of each pair of eyes; or lotions in 50% alcohol were applied to the shaved skin. These lotions were applied also to the unbroken skin of the forearm of 3 human volunteers daily for 4 weeks.

RESULTS

Antifungal Activities

Effect of Chain-length and End-group.—The relationship between length of polymethylene chain in the BIQ series and ability to inhibit growth of a strain of *Trichophyton mentagrophytes* is expressed in Fig. 1. There is a fairly sharp rise in potency as chain-length increases from 10 to 14 methylene groups. With further increase in chain-length, potency at 3 days falls gradually; but at 14 days differences between most of the chain-lengths have vanished, giving a plateau of higher activity extending from BIQ 14 to BIQ 19. The bisquinolinium compounds were almost equiactive to the corresponding bis*iso*quinolinium against the same strain of *T. mentagrophytes*, the effect of altering chain-length being therefore strictly comparable. For example, in 5 experiments with this fungal strain in which their activities were compared directly, the MICs of BIQ 14 iodide were 1.89 μg./ml. at 3 days and 5.74 μg. at 14 days, and of BQ 14 iodide 1.89 and 3.79 μg. respectively. In comparable tests with other drugs, the MICs in μg./ml. at 3 and 14 days were: dibromopropamidine isethionate, 160 and 640; undecylenic acid, 40 and 80; cetrimide, 12.5 and 25.

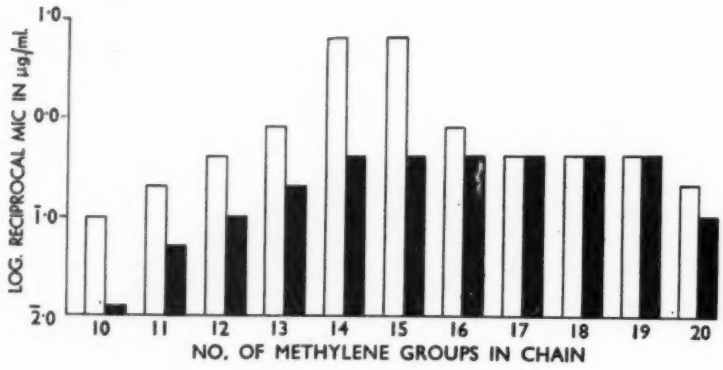

FIG. 1.—Relation of number of methylene groups in chain of bis*iso*quinolinium compounds to activity against *T. mentagrophytes* in Sabouraud's broth at 27° C. White, after 3 days' incubation; black, after 14 days'.

Action Against Other Strains and Species.—Results obtained with the one strain of *T. mentagrophytes* justified further investigations of the more active compounds. For this, BIQ 14, BIQ 16, BQ 14, and BQ 16 were selected for reasons discussed below. The results of *in vitro* tests of the activities of these compounds against a number of strains and species of fungi that attack skin are summarized in Table I, from which it will be seen that all four potently inhibited all strains of fungi studied. The compounds showed approximately equal activities, except against *Candida albicans*, which was more sensitive to the hexadecamethylene chain-length. Again for reasons discussed below, BIQ 16 was selected for further study.

Antagonism of Antifungal Activity.—Table II shows that sera and hair exerted a slight antagonistic action upon the effect of BIQ 16 on *T. mentagrophytes*. A comparable effect was also seen when the conditions of the experiment were varied, in respect of either the serum employed (bovine, rabbit), the hair (bovine, dog), the inhibitory compound (BIQ 14) or the species of fungus (*T. verrucosum*). Table II also shows that bovine bile was the most potent antagonist: this was used in tests of fungicidal activity.

TABLE I

ACTIVITIES OF BIQ 14, BIQ 16, BQ 14 AND BQ 16 IODIDES AGAINST SOME FUNGI INFECTING SKIN

Minimal inhibitory concn. (μg./ml.) after 14 days' incubation in Sabouraud's broth at 27° C. In parentheses, number of strains examined.

Species	BIQ 14	BIQ 16	BQ 14	BQ 16
Candida albicans	5·0	1·25	7·0	1·8
Microsporum Audouini ..	1·25	0·625	1·25	0·44
Microsporum canis (2) ..	1·25–5	1·25	1·25–2·5	1·25
Trichophyton mentagrophytes (5)	1·25–10	0·625–5	0·625–5	0·3125–5
Trichophyton rubrum	2·6	2·5	1·8	1·8
Trichophyton verrucosum ..	0·44	0·44	0·44	0·3125

TABLE II

EFFECT OF VARIOUS MATERIALS ON THE INHIBITION OF GROWTH OF *T. MENTAGROPHYTES* BY BIQ 16 METHOSULPHATE IN SABOURAUD'S BROTH AT 27° C.

Material added to Medium		MIC (μg./ml.) at 14 days
Nature	% Concn.	
Thymine	0·1	1·25
Uracil	0·1	2·5
Human hair	—	10
Human serum	10	5
Horse serum	10	10
"Tween 80"	0·5	10
"Lubrol"	0·5	20
Egg lecithin	0·1	80
Vegetable lecithin	0·1	125
Dried bovine bile	2	4,000
None		1·25

Fungicidal Action.—After exposing spores of *T. mentagrophytes* to 25 mg. phenol/ml. saline at 20° C. for 10 min., viable spores could not be detected on subculture. On the other hand, in similar conditions, spores remained viable after exposure to 50 mg. BIQ 16 methosulphate/ml. saline for 24 hr. The fungicidal action of BIQ 16, however, was readily demonstrated when spore suspensions were incubated in broth at 27° C., as will be seen from the experiment illustrated in Table III. Subcultures were negative after 7 days' incubation of spores in 1.25 μg./ml. of BIQ 16 methosulphate, which is approximately the MIC. From this and comparable experiments with BIQ 14, it was concluded that these compounds are highly fungicidal when acting in nutrient medium for several days.

TABLE III

FUNGICIDAL ACTION AGAINST *T. MENTAGROPHYTES* OF BIQ 16 METHOSULPHATE AT 27° C.

Time of Incubation of Spore Suspension	Minimal Fungicidal Concn. (μg./ml.)*
5 min.	>640
1 hr.	>640
1 day	160
2 days	80
7 days	1·25

* Lowest concn. at which subcultures in 2% bile broth failed to grow.

Toxicity

Acute.—In mice the acute subcutaneous LD50 values (mg./kg.) for the iodides were:

BIQ 14, 24 ± 1.1; BIQ 16, 29 ± 1.6; BQ 14, 16 ± 2.3; and BQ 16, 18 ± 0.5. The acute oral LD50 values (mg./kg.) for the methosulphates were: BIQ 14, 228 ± 13; BIQ 16, 275 ± 19.

In a series of intradermal injections in rabbits, a solution containing 80 μg. BIQ 14 iodide/ml. saline caused erythema followed by necrosis and healing in 4 of 9 injection sites and a similar solution of BIQ 16 caused similar reactions in 5 of 9 sites. When the solutions of either compound were diluted to half this concentration, reactions were observed in none of 9 sites with BIQ 14 and in 1 of 9 with BIQ 16.

Subacute.—Solutions containing 1 mg. BIQ 14 or BIQ 16 methosulphate/ml. saline appeared to be without effect on the eyes of rabbits when instilled daily over a period of two weeks.

When lotions containing 5 or 2.5 mg. BIQ 16 methosulphate/ml. 50% aqueous alcohol were applied daily to the shaved skins of rabbits over a period of 4 weeks, no reaction to the drug was seen, although the alcoholic medium caused slight temporary flushing. On the other hand a lotion of 5 mg. BIQ 14 methosulphate/ml. aqueous alcohol caused marked erythema and its application was discontinued after 4 days; a lotion containing 2.5 mg./ml. was tolerated. When a lotion containing 5 mg. BIQ 16 iodide/ml. aqueous alcohol was applied daily to the unbroken skin of 3 human subjects, no reactions were seen.

DISCUSSION

There was little to choose in antifungal potency between *iso*quinolinium and quinolinium compounds of the same chain-length; but chain-length itself had a considerable effect. The contour of the relation between chain-length and potency for *T. mentagrophytes* (Fig. 1) most nearly resembles that for *Mycobacterium phlei* among bacteria previously examined (Collier *et al.*, 1953). This provides another example of compounds possessing similar antifungal and antimycobacterial properties previously only observed in thioureas by Mayer, Eisman, and Konopka (1953).

The low fungicidal activity of BIQ 16 against spore suspensions in saline was not unexpected in view of the failure of Klarmann and Wright (1954) to detect fungicidal activity in similar tests with conventional quaternary ammonium antibacterials. The striking increase in fungicidal activity with continued exposure to broth may explain the difference in shape of the chain-length-potency contours illustrated in Fig. 1. Readings after 3 days' incubation might measure growth inhibition and those after 14 days' might measure fungicidal activity. These two distinct forms of action might be expected to show different relations to compound chain-length.

TABLE IV

POLYMETHYLENE BISQUINOLINIUM AND BIS*ISO*QUINOLINIUM IODIDES

The reaction solvent was methylethylketone.

$$\left\{R^+-[CH_2]_n-R^+\right\}2\,I^-$$

Value of *n*	Reaction Time (hr.)	M.p.	Cryst. Form	Crystn. Solvent	Found %*				Formula	Required %			
					C	H	N	I		C	H	N	I
Quinolinium													
11	104	190–192°	Yellow needles or rosettes	EtOH	52·5	5·4	3·9	37·8	$C_{29}H_{36}N_2I_2$	52·25	5·45	4·2	38·1
12	180	Rapid: 157–9° Slow: sinters at 157–9° m.p. 186–188°	Yellow needles	EtOH Et_2O	53·0	5·8	4·1	37·7	$C_{30}H_{38}N_2I_2$	52·9	5·6	4·1	37·35
13	180	170°	Yellow granules	,,	53·55	5·7	4·0	35·9	$C_{31}H_{40}N_2I_2$	53·6	5·8	4·0	36·6
14	300	157–158°	,, ,,	,,	54·4	6·0	4·05	35·8	$C_{32}H_{42}N_2I_2$	54·2	6·0	3·95	35·9
16	160	148–150°	,, ,,	,,	55·1	6·55	3·8	34·3	$C_{34}H_{46}N_2I_2$	55·4	6·3	3·8	34·5
18	120	157–158°	Yellow nodules or granules	,,	56·7	6·6	3·6	33·3	$C_{36}H_{50}N_2I_2$	56·5	6·6	3·7	33·25
20	240	160–161°	Yellow granules	,,	57·5	6·7	3·3	31·7	$C_{38}H_{54}N_2I_2$	57·6	6·9	3·5	32·1
*Iso*quinolinium													
14†	48	163–165°	White nodules	,,	62·2	7·1	4·6	26·1‡	$C_{32}H_{42}N_2Br_2$	62·5	6·9	4·6	26·1‡
15	24	145–146°	Yellow nodules	,,	55·0	6·0	4·0	34·5	$C_{33}H_{44}N_2I_2$	54·85	6·1	3·9	35·2
17	24	85–86°	Yellow prisms	MeCOMe/ Et_2O	56·3	6·65	3·6	33·8	§$C_{35}H_{48}N_2I_2$	56·0	6·45	3·7	33·9
19	48	133–134°	Yellow needles	EtOH/Et_2O	56·4	6·9	3·5	32·8	$C_{37}H_{52}N_2I_2$	57·1	6·7	3·6	32·65

* Dried at 100° *in vacuo*. † Bromide (this was also prepared by double decomposition from the methosulphate). ‡ Bromine.
§ Dried at 56° *in vacuo*.

The compounds BIQ 14 and 16 and BQ 14 and 16 were chosen for further examination, for three reasons: (i) they came close to the peaks of activity against *T. mentagrophytes*; (ii) compounds with an even number of methylene groups were distinctly easier to prepare than the odd, especially those with 15, 17, or 19 methylene groups; and (iii) in the even members of both series acute subcutaneous toxicity in mice increased sharply above the hexadecamethylene compounds. Of these four compounds, BIQ 14 and 16 appeared preferable on the grounds of lower toxicity and greater ease of preparation. The same considerations indicated that BIQ 16* was preferable to BIQ 14. BIQ 16 had the additional advantages of greater activity against *Staphylococcus aureus* and other bacteria that may infect skin (Collier *et al.*, 1953) and against *Candida albicans*. It may be noted in passing that the hexadecamethylene compounds provide an exception to the usual trend towards increased toxicity which accompanies increase in chain-length. When the data obtained in examining the toxicities of BIQ 14 and 16 were considered with those for *in vitro* antifungal potencies, clinical trials seemed justified. The result of a preliminary trial of BIQ 14 in calves was encouraging; trials of BIQ 16 are now in progress.

*Registered name "Teoquil."

CHEMICAL SECTION

Microanalyses were by Drs. Weiler and Strauss, Oxford. All melting-points are uncorrected.

Most of the polymethylene di-iodides used in this work have already been described, but the following appear to be new: *pentadecamethylene di-iodide*, flat glistening white needles, m.p. 49–50.5°, from alcohol. (Found: C, 39.1; H, 6.5; I, 54.4. $C_{15}H_{30}I_2$ requires C, 38.8; H, 6.5; I, 54.7%); *heptadecamethylene di-iodide*, feathery white needles, m.p. 56–57.5°, from alcohol. (Found: C, 41.8; H, 7.0; I, 51.3. $C_{17}H_{34}I_2$ requires C, 41.5; H, 7.0; I, 51.6%); and *nonadecamethylene di-iodide*, glistening white plates, m.p. 63–64°, from alcohol. (Found: C, 43.8; H, 7.3; I, 48.6. $C_{19}H_{38}I_2$ requires C, 43.85; H, 7.4; I, 48.85%.)

1:17-*Dicyanoheptadecane*, an intermediate in the synthesis of nonadecamethylene di-iodide, is also apparently unrecorded. This crystallized from light petroleum (b.p. 40–60°) in small white leaflets, m.p. 54–55°. (Found: C, 78.4; H, 11.7; N, 9.75. $C_{19}H_{34}N_2$ requires C, 78.6; H, 11.8; N, 9.7%.)

Some of the bis*iso*quinolinium salts investigated have been described in our earlier paper (Collier *et al.*, 1953). The new *polymethylene bisquinolinium* and *bisisoquinolinium bromides* and *iodides*, the properties of which are given in Table IV, were prepared by the general method previously described (Collier *et al.*, 1953). As a rule, the *iso*quinolinium salts were more readily purified, and obtained in greater yield, than were the quinolinium compounds.

Other salts were prepared by double decomposition as indicated below.

*Tetradecamethylene bis(*iso*quinolinium methosulphate).*—Tetradecamethylene bis(*iso*quinolinium iodide) [10 g.] and dimethyl sulphate (6 ml.) were heated together at 100° for 15 min. Acetone (200 ml.) was then added, and the mixture refluxed for 30 min. The white product was filtered and recrystallized from a mixture of alcohol and acetone, yielding the required *methosulphate* as white prisms, m.p. 224–225°. (Found: C, 60.2; H, 6.7; N, 4.2; S, 9.4. $C_{34}H_{48}O_8N_2S_2$ requires C, 60.4; H, 7.2; N, 4.1; S, 9.5%.)

Similarly, *tetradecamethylene bis(quinolinium methosulphate)* was obtained as tan-coloured microcrystals of the monohydrate, m.p. 212–213°, from alcohol-ether. (Found: C, 58.9; H, 6.9; N, 4.0; S, 8.95. $C_{34}H_{48}O_8N_2S_2$. H_2O requires C, 58.8; H, 7.3; N, 4.0; S, 9.2%.)

Similarly, *hexadecamethylene bis(*iso*quinolinium methosulphate)* was obtained as clusters of small white needles, m.p. 172°, from alcohol-acetone. (Found: C, 61.0; H, 7.4; N, 3.8; S, 9.1. $C_{36}H_{52}O_8N_2S_2$ requires C, 61.4; H, 7.45; N, 4.0; S, 9.1%.)

*Tetradecamethylene bis(*iso*quinolinium nitrate).*—A solution of the corresponding methosulphate (20 g.) in water (650 ml.) was treated with powdered potassium nitrate (120 g.) with stirring. The white crystalline precipitate was filtered off, dried and recrystallized from alcohol-ether, giving the required *nitrate* as white needles, m.p. 128–129°. (Found: C, 66.3; H, 7.4; N, 9.8. $C_{32}H_{42}O_6N_4$ requires C, 66.4; H, 7.3; N, 9.7%.)

*Tetradecamethylene bis(*iso*quinolinium chloride)* was obtained from the methosulphate in a similar manner, and the dihydrate crystallized from a mixture of alcohol, acetone, and ether as white nodules, m.p. 103–104°. (Found: C, 68.0; H, 8.25; N, 4.8; Cl, 12.65. $C_{32}H_{42}N_2Cl_2$. $2H_2O$ requires C, 68.4; H, 8.3; N, 5.0; Cl, 12.7%.) On drying *in vacuo* at 100°, the anhydrous salt, m.p. 133–134°, was obtained.

*Tridecamethylene bis(*iso*quinolinium perchlorate)* was prepared from a solution of the corresponding iodide (0.5 g.) in warm alcohol (10 ml.) by treatment with 60% aqueous perchloric acid (0.5 g.). The oil, which separated immediately, solidified on scratching to a pale yellow powder, which on recrystallization from methanol-ether yielded the required *perchlorate* as a white micro-crystalline powder, m.p. 128–129°. (Found: C, 58.4; H, 6.2; N, 4.3; Cl, 11.2. $C_{31}H_{40}O_8N_2Cl_2$ requires C, 58.2; H, 6.3; N, 4.4; Cl, 11.1%.)

It was originally proposed to extend this series by preparing polymethylene bisquaternary salts of various alkyl substituted quinolines and *iso*quinolines. Thus, 6-methylquinoline, on refluxing with tetradecamethylene di-iodide in methyl ethyl ketone, yielded *tetradecamethylene bis(6-methylquinolinium iodide)* as yellow-brown prisms, m.p. 133–135°, from methanol-acetone-ether (Found: C, 55.9; H, 6.2; N, 3.6; I, 34.1. $C_{34}H_{46}N_2I_2$ requires C, 55.4; H, 6.3; N, 3.8; I, 34.5%), and 3-methyl*iso*quinoline gave *tetradecamethylene bis(3-methyl*iso*quinolinium iodide) as* pale yellow micro-crystals, m.p. 227–228°, from methanol-ether. (Found: C, 55.4; H, 6.6; N, 3.9; I, 34.55%.)

When, however, tetradecamethylene di-iodide was refluxed with (*a*) quinaldine, (*b*) 7-methylquinoline or (*c*) 1-methyl*iso*quinoline in methyl ethyl ketone, the resultant quaternary salts gave analytical figures corresponding to the loss of two methylene groups from the expected products as indicated below:

(*a*) m.p. 171–172°. Found: C, 54.0; H, 6.1; N, 4.1; I, 35.8.

(*b*) m.p. 160–161°. Found: C, 54.0; H, 6.2; N, 3.9; I, 35.7.

(*c*) m.p. 178–179°. Found: C, 53.9; H, 6.3; N, 3.9; I, 35.8. $C_{32}H_{42}N_2I_2$ requires C, 54.2; H, 6.0; N, 3.95; I, 35.9%.

Obviously, if this had been due to a simple demethylation of the nuclear methyl group, products (*a*) and (*b*) would have had the same melting-point and would have been identical with the previously prepared tetradecamethylene bisquinolinium iodide, and product (*c*) would have been the corresponding bis-*iso*quinolinium iodide. Mixed melting-point determinations showed that this was not so.

No simple explanation of these anomalous results is apparent, but no extensive study of the problem has been made. The alkylquinolines and -*iso*quinolines were purified before use by either recrystallization of a solid derivative or by redistillation. The purified bases all yielded picrates of the correct melting-point.

Summary

1. Polymethylene bis*iso*quinolinium compounds with 15, 17, and 19 methylene groups and a series of bisquinolinium salts with 11–14, 16, 18, and 20 methylene groups have been prepared.

2. Polymethylene bis*iso*quinolinium salts with 10–20 methylene groups and the corresponding quinolinium salts were examined for activity against *T. mentagrophytes*. In both series, activity increased with increase in chain-length up to the tetradecamethylene member: a marked decline in activity was seen at the eicosane compounds.

3. The tetradeca- and hexadecamethylene members of both series were found to inhibit 11 strains of 6 species of pathogenic fungi in Sabouraud's broth at concentrations between 0.3 and 10 μg./ml.

4. The tetradeca- and hexadecamethylene bis*iso*quinolinium salts were slightly antagonized by hair and serum and the latter compound was potently antagonized by bovine bile.

5. Exposure for 24 hr. at 20° C. to hexadecamethylene bis(*iso*quinolinium methosulphate) did not destroy spores of *T. mentagrophytes* suspended in saline. On the other hand, viable spores could not be detected after incubation for 7 days at 27° C. with a concentration of 1.25 μg./ml. of this compound in Sabouraud's broth.

6. The acute subcutaneous toxicities of the four tetradeca- and hexadecamethylene compounds and the acute oral toxicities of the two *iso*quinolinium compounds were determined in mice. Observations were also made on the acute intradermal toxicities and on the subacute toxicities to eyes and skin of rabbits.

7. The results appeared to warrant clinical trial of the tetradeca- and hexadecamethylene bis*iso*-quinolinium compounds in fungal infections of the skin.

We are indebted to Dr. R. W. Riddell for some of the strains of fungi used, to Mr. C. J. Eastland for preparation of lotions, to Miss S. Lewis for assistance in toxicity tests, and to the directors of Messrs. Allen and Hanburys Ltd. for permission to publish this work.

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THE ACTION OF ESERINE ON THE BLOOD PRESSURE OF THE RAT

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(RECEIVED MAY 10, 1955)

Recently α-cocaine was prepared in this department under the direction of Dr. H. R. Ing by Dr. R. Foster. In the course of pharmacological experiments on the action of α-cocaine, which will be described in another paper, it was decided to test its action on the pressor effect of adrenaline when eserine had been given.

The experiments were to be performed on cats, but as the supply of cats was short the observations were made in rats. It was then found that eserine had a pressor action in the rat, and the experiments now described were undertaken to analyse this action. During the course of the work a paper appeared by Dirnhuber and Cullumbine (1955) which also describes the pressor effect of certain anticholinesterase agents in the rat. Since my observations confirm many of their findings, attention will be given mainly to new points or points of difference from them.

METHODS

In general, the method for recording the rat's blood pressure described by Crawford and Outschoorn (1951) has been followed. Rats (200–400 g.) of both sexes were used, and anaesthetized with 0.7 ml. per 100 g. of body weight of 25% urethane solution subcutaneously. To record the blood pressure a cannula was inserted into the carotid artery and connected with a mercury manometer, as described in detail by Condon (1951). A small polythene cannula, 0.5 mm. in diameter, was inserted into the jugular vein, and was used for injecting drugs. Before the experiment was started 1–1.5 mg./100 g. of heparin was injected. All doses of drugs were injected in 0.1 ml. and washed in with the same volume of saline. In most of the experiments artificial respiration was applied. The effect of eserine was the same no matter whether the animal was breathing naturally or not.

The hindlegs of rats were perfused by inserting a cannula into the aorta immediately before the bifurcation. The perfusion fluid was oxygenated Locke solution. In a few experiments 25% "Intradex" in Locke solution was used. The venous outflow was recorded by means of Stephenson's recorder (1948).

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RESULTS

Action of Different Anticholinesterase Agents.—The effect of the intravenous injection of 10 μg. eserine sulphate into a rat of 230 g. weight is shown in Fig. 1C. This amount of eserine, equivalent to 43 μg./kg., caused a rise of pressure equal to 50 mm. Hg which lasted for about 20 min. Such an effect was observed in 60 experiments varying from one rat to another only in duration; the longest duration was 45 min. and the shortest was 6 min. Dirnhuber and Cullumbine observed similar effects with sarin (*iso*propylmethylphosphonofluoridate), dyflos (di*iso*propylphosphorofluoridate), TEPP (tetraethylpyrophosphate) and E.600 (diethyl-*p*-nitrophenylphosphate). With other anticholinesterase agents I failed to observe the pressor action. Thus in Fig. 1A the injection of 25 μg. Nu 683 (the dimethylcarbamate of 2-hydroxy-5-phenylbenzyltrimethylammonium bromide) caused a fall of pressure. Similarly in Fig. 1B the injection of 25 μg. Nu 1250 (*N*-*p*-chlorophenyl-*N*-methylcarbamate of *m*-hydroxyphenyltrimethylammonium bromide) failed to cause a rise of pressure. Finally, in Fig 1D the injection of 10 μg. BW284C51 (the dimethobromide of 1:5-di-(*p*-*N*-allyl-*N*-methylaminophenyl)-pentan-3-one) caused an insignificant rise. Neostigmine (Prostigmin methylsulphate) was also ineffective in causing a rise of pressure which was at all comparable with that produced by eserine. In three experiments the injection of 10 μg. neostigmine caused a fall of blood pressure. In one of these the injection of 30 μg. neostigmine caused a rise of 13 mm. and the injection of 100 μg. caused a rise of 20 mm. In a fourth experiment the injection of 30 μg. caused a rise of 22 mm., but a repetition of the injection caused a fall.

Action of Nicotine.—The pressor effect of eserine was found to be abolished in some rats by nicotine as shown in Fig. 2. At the arrow 10 μg. eserine was injected into a rat of 225 g. and the injection produced the usual rise. At 1, 50 μg.

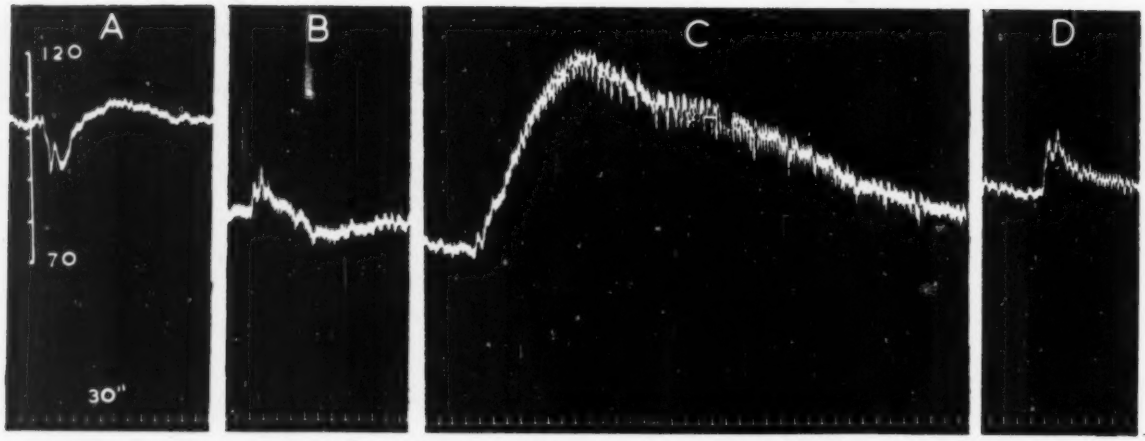

FIG. 1.—Rat, 230 g., under urethane. The action of various anticholinesterases on arterial blood pressure. A, 25 μg. Nu 683 i.v. B, 25 μg. Nu 1250 i.v. C, 10 μg. eserine i.v. D, 10 μg. BW284C51 i.v. Note that only eserine caused an appreciable rise of blood pressure.

nicotine acid tartrate was injected, causing a transient rise of pressure ; at 2, 100 μg. nicotine was injected ; at 3 and 4, 1 mg. nicotine was injected. The injection of 10 μg. eserine then failed to cause any rise when given twice, and 30 μg. eserine was also inactive. Thus the total of 9.5 mg./kg. of nicotine abolished the action of eserine. The same result was obtained in two other experiments, the amounts of nicotine injected being equivalent to 6.2 and 11.8 mg./kg. respectively. In three other experiments injections of nicotine made at longer intervals failed to abolish the action of eserine although the total amount was 3.55, 1.3, and 3.55 mg./kg. respectively. However, when relatively small amounts of hexamethonium were given in addition, namely, 1.73, 7.6, and 3.8 mg./kg., the pressor effect of eserine was abolished.

Action of Hexamethonium.—Dirnhuber and Cullumbine stated that sarin caused a rise of pressure in the presence of amounts of hexamethonium equivalent to 10–20 mg./kg. ; after large doses of hexamethonium from 200–400 mg./kg., sarin then had no effect. In my experiments with eserine large amounts of hexamethonium (128, 142, and 167 mg./kg. in three experiments) given in one or two injections abolished the pressor action of amounts of eserine in the range of 30–90 μg./kg. ; but amounts of eserine such as 1.5 mg./kg. were still active.

The effect of hexamethonium in abolishing the pressor effect of eserine was much greater after adrenalectomy. For example, in two adrenalectomized rats 1.43 and 5.0 mg./kg. were sufficient to abolish the pressor effect. In two other rats 3.8 and 4.2 mg./kg. hexamethonium did not reduce the action of eserine before adrenalectomy ; after adrenalectomy, however, the injection of 2.8 and 4.2 mg./kg. abolished the pressor effect. A similar effect was observed by Dirnhuber and Cullumbine when investigating sarin.

In the experiment shown in Fig. 3 the effect of 30 μg. eserine shown in A was reduced by injection of 0.8 mg. C6 to that shown in B. In the course of the next 70 min. a series of 11 injections of 0.4 mg. C6 were given, and the effect of 30 μg. eserine was increased to the point shown in C. Injections of nicotine were then given up to a total of 9.85 mg. These did not modify the action of eserine as shown in D.

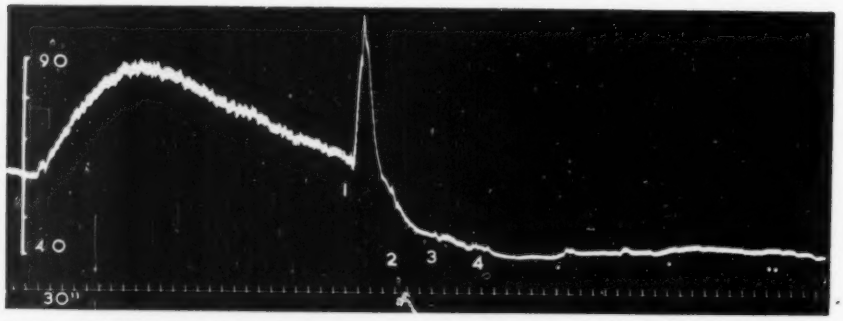

FIG. 2.—Rat, 225 g., under urethane. The action of nicotine on the pressor effect of eserine injected i.v. At one white spot, 10 μg. eserine. At 1, 50 μg. nicotine acid tartrate. At 2, 100 μg. nicotine acid tartrate. At 3 and 4, 1 mg. nicotine acid tartrate. At two white spots, 30 μg. eserine. Note complete abolition of the effect of eserine after nicotine.

Potentiation by Cocaine.—The pressor effect of eserine was increased by cocaine as shown in Fig. 4. In Fig. 4A is shown the rise of pressure produced by 5 μg. eserine ; 0.75 mg. cocaine hydrochloride was then injected into the peritoneal cavity. The response

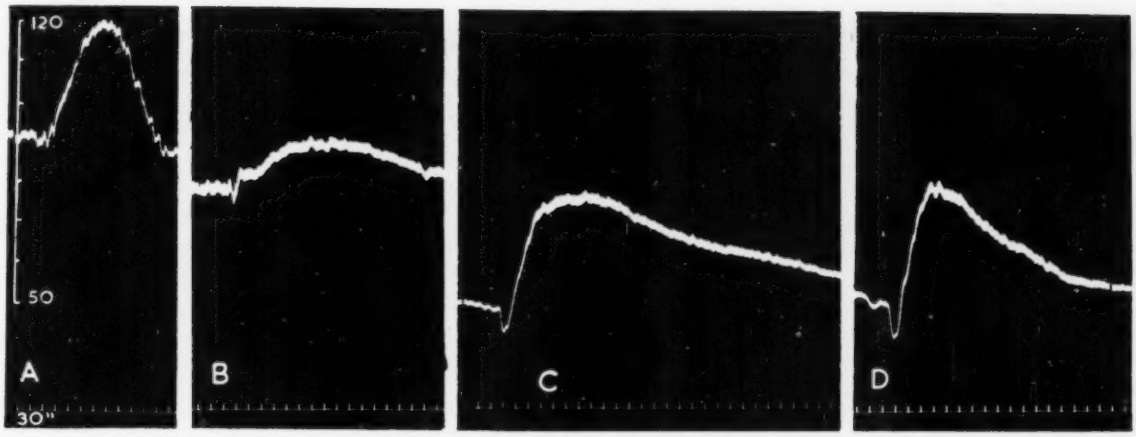

FIG. 3.—Rat, 230 g., under urethane. The action of hexamethonium and nicotine on the pressor effect of eserine. At A, B, C and D, injection of 30 μg. eserine. Between A and B 0.4 mg. hexamethonium was injected twice. Between B and C 0.4 mg. hexamethonium was injected eleven times. Between C and D 9.85 mg. nicotine acid tartrate was injected. Note decrease of the effect of eserine after small doses of hexamethonium, and return of the effect after larger doses. Note also that after hexamethonium nicotine did not alter the effect of eserine.

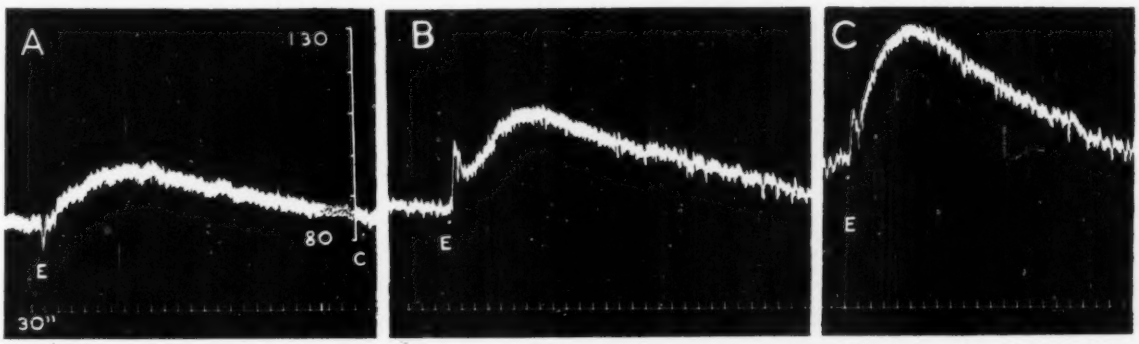

FIG. 4.—Rat, 260 g., under urethane. The action of cocaine hydrochloride on the pressor effect of eserine. At E in A, B and C, 5 μg. eserine was injected i.v. At C in A, 0.75 mg. cocaine hydrochloride intraperitoneally. B was taken 20 min. and C 45 min. after injection of cocaine. Note the potentiation of the pressor effect of eserine by cocaine.

to 5 μg. eserine increased as shown in Fig. 4B, which was 20 min. later, and further increased in Fig. 4C, which was 45 min. later.

Effect of Decerebration.—Eserine (30 μg.) caused a rise of blood pressure in two rats which were decerebrated; the result in one of these is shown in Fig. 5, from which it is evident that the rise was large. In both experiments the natural respiration stopped after the injection, and although respiration was then maintained artificially the blood pressure fell and further injections of eserine were without effect. In spinal rats, eserine had very little effect on the blood pressure, but produced a small rise when doses as large as 0.5 mg. were injected.

Abolition of Response by Dibenamine.—Dirnhuber and Cullumbine found that the pressor effect

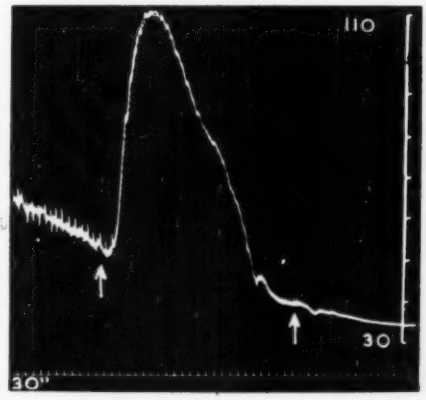

FIG. 5.—Rat, 320 g., decerebrated. At white spot 10 μg. eserine was injected. At arrow 30 μg. eserine was injected. After first dose of 30 μg. eserine the natural respiration stopped and had to be continued artificially. Observe the absence of the effect of eserine at the second injection.

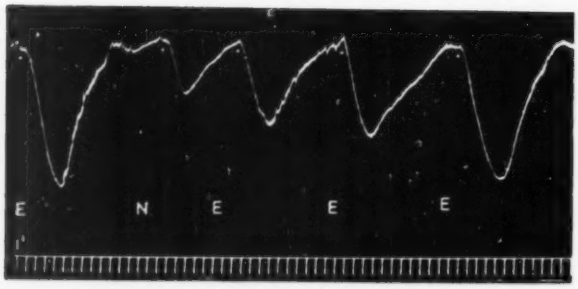

FIG. 6.—Perfusion of rat's hindlegs. The venous outflow recorded by Stephenson's recorder. At E, 1 mg. eserine; at N, 1 mg. nicotine acid tartrate, injected into cannula. Observe the reduction of the effect of eserine after nicotine.

of sarin was reduced to a brief initial spike in the presence of ergotamine or tolazoline. I have found that 1 mg. dibenamine abolished the pressor action of eserine completely. I also observed, in confirmation with Dirnhuber and Cullumbine, that the pressor effect was abolished by atropine, but was not appreciably affected by adrenalectomy or by evisceration. The response was also unaltered by section of the splanchnic nerves.

Peripheral Constrictor Action of Eserine.—To determine whether eserine exerted any part of its pressor effect by a direct action on the vessels, experiments were performed in which the hindlegs of rats were perfused through a cannula tied in the aorta. It was found that when eserine in doses of 1 mg. was injected into the cannula it produced a diminution in venous outflow as shown in Fig. 6. This constrictor action was diminished by an injection of 1 mg. nicotine, and then was restored to its original size by successive injections. The constrictor effect was observed in 11 experiments.

Eserine was not found to affect the rate or amplitude of the isolated rat heart when injected in amounts up to 0.1 mg.

DISCUSSION

The observations described in this paper give general support to the conclusions of Dirnhuber and Cullumbine concerning the mechanism of the pressor effect of sarin in the rat, which they found was also exerted by eserine, dyflos, TEPP, and E.600. While they state that the doses of these substances required to cause a rise of blood pressure were "near-lethal," I have regularly obtained large rises of pressure with 10–30 μg. eserine. The rise was obtained in the decerebrate rat as well as in the rat under urethane. A small rise was also obtained in the spinal rat as Dirnhuber and Cullumbine found with sarin. The main effect on the central nervous system seems, nevertheless, to be exerted at points not lower than the medulla. In agreement with Dirnhuber and Cullumbine the rise of pressure is abolished by atropine, this substance probably acting centrally, and also by hexamethonium and nicotine, from which it may be concluded that the impulses producing the rise of blood pressure pass through the sympathetic ganglia. The rise is augmented by cocaine and absent after the injection of dibenamine, as would be expected if it were due to sympathetic impulses arriving in the walls of the blood vessels.

Liberation of adrenaline and noradrenaline from the adrenal glands appears to play a part in causing the rise of pressure. As long ago as 1921 Stewart and Rogoff observed that after giving eserine to cats the output of adrenaline from the adrenals was increased 10 to 15 times. I found that after the removal of the adrenals much smaller amounts of hexamethonium were sufficient to abolish the rise of blood pressure due to eserine. In the second place, in some experiments, the effect of eserine was depressed as usual by the first injection of hexamethonium, but increased with subsequent injections of hexamethonium as shown in Fig. 3. This change was probably explained by the augmentation of the pressor effect of adrenaline which has been observed by various workers (Slater and Dresel, 1952) to be produced by hexamethonium. It would seem that much larger amounts of hexamethonium are required to block the adrenal glands than to block the sympathetic ganglia, and the effect of the small amount of adrenaline still liberated from the glands due to incomplete block is steadily increased by further injections of hexamethonium.

The augmentation of the pressor effect by cocaine gave further support to the view that the effect was due in part to the release of adrenaline or noradrenaline.

Finally, I observed a constrictor effect of eserine in the hindlegs of the rat perfused with Locke solution through the aorta. This effect was diminished by the injection of nicotine which itself produced constriction; the effect would therefore seem to resemble the constrictor effect of nicotine recently described by Kottegoda (1953) in the perfused rabbit ear. Ambache and Lessin (1955) have also found evidence of a "neuronal" action of eserine in isolated strips of rabbit ileum. Thus, not only in centres in the brain but also at points of the peripheral nervous system, eserine has a stimulant action which is unusually strong in the rat.

Dirnhuber and Cullumbine found that the pressor effect of sarin was shared by eserine, dyflos, TEPP, and E.600. I have observed that the effect

was not obtained with substances containing quaternary nitrogen atoms such as neostigmine, Nu 1250, Nu 683, and BW284C51. This suggests that the former compounds are active because they penetrate the central nervous system more readily.

Summary

1. Small amounts of eserine (10 μg.) caused an appreciable rise of blood pressure in the rat anaesthetized by urethane or decerebrated.

2. In some spinal preparations, also, large amounts (0.5 mg.) caused a rise.

3. This action was not shared by neostigmine or other anticholinesterases containing a quaternary nitrogen atom.

4. The action of eserine was blocked by nicotine and by large amounts of hexamethonium. After adrenalectomy it was blocked by small amounts of hexamethonium.

5. The action was potentiated by cocaine, and blocked by dibenamine.

6. Eserine had some constrictor action in the perfused hindlegs of the rat; this action was depressed by nicotine.

7. The action appeared to be mainly due to the discharge of impulses from a centre or centres in the brain.

I am deeply indebted to Professor J. H. Burn for his guidance and encouragement throughout this work.

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ANTAGONISM OF ACETYLCHOLINE BY (+)- AND (−)-HYOSCYAMINE

BY

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(RECEIVED MAY 10, 1955)

Atropine is generally accepted as a competitive antagonist of acetylcholine (ACh) at cholinergic nerve endings. Quantitative investigation of the antagonism of ACh contractions of isolated guinea-pig ileum by atropine, however, indicated that the antagonism was non-competitive. This observation confirmed similar studies of ACh-atropine antagonism reported by Schild (1947). In a recent paper (Marshall, 1955) a compound was reported of which the (−)-isomer is a competitive and the (+)-isomer a non-competitive antagonist of histamine. It was thought that this might also apply to (+)- and (−)-hyoscyamine, antagonizing ACh, and that atropine, or (±)-hyoscyamine, might thus be predominantly non-competitive. The competition between these isomers and ACh has accordingly been investigated.

METHODS

Guinea-pig ileum was suspended in oxygenated Tyrode solution at 37° C. in an automatic organ bath of the type described by Boura, Mongar and Schild (1954). Determinations of pA_2 and pA_{10} against ACh contractions were made on samples of atropine and of (+)- and (−)-hyoscyamine by the method of Schild (1947). The nature of the antagonism was assessed as described by Marshall (1955).

RESULTS

Table I shows the pA values and type of antagonism of ACh by the three compounds examined.

The results show that, whereas ACh antagonism by atropine and (+)-hyoscyamine is non-competitive, that by (−)-hyoscyamine is competitive. The (−)-isomer has the most potent anti-ACh action, and the potency of the racemic compound, atropine, lies between those of the two optical isomers. These results agree with those previously reported for an optically active antihistamine compound (Marshall, 1955), and offer an explanation of the non-competitive antagonism found in atropine. Since atropine is a mixture of the two optical isomers—one competitive and the other non-competitive—its antagonism against ACh might be expected to mediate between the two types. In subsequent experiments using guinea-pig ileum in Tyrode solution containing sodium acetate instead of glucose, and with rat ileum in both glucose and acetate Tyrode, I have found atropine to be competitive, though the results were always on the border-line between the two types of antagonism.

TABLE I

ANTAGONISM OF ACh CONTRACTIONS OF GUINEA-PIG ILEUM

(ACh concentrations: 0·01, 0·02, 0·1 μg./ml.)

Antagonist	pA_2	pA_{10}	(pA_2-pA_{10})	Significance of Deviation from $(pA_2-pA_{10})=0.95$ $(P=0.05)$
Atropine ..	8·82	8·12		
	8·77	8·12		
	8·76	8·22		
	9·02	8·05		+(Non-competitive)
	8·89	8·10		
	8·76	8·06		
	Mean 8·84	Mean 8·11	0·73	
(+)-Hyoscyamine	7·79	7·39		
	7·95	7·12		
	7·76	7·12		
	7·97	7·08		+(Non-competitive)
	7·78	7·02		
	7·83	7·11		
	Mean 7·85	Mean 7·14	0·71	
(−)-Hyoscyamine	9·67	8·32		
	9·10	8·38		
	9·32	8·45		
	9·37	8·68		−(Competitive)
	9·45	8·67		
	9·20	8·43		
	9·36	8·44		
	Mean 9·35	Mean 8·48	0·87	

DISCUSSION

These observations offer a possible explanation of the variable results, with regard to type of

antagonism, obtained previously between histamine and four samples of promethazine (Marshall, 1955). Since all the samples were shown to be optically inactive, and therefore racemic mixtures, the (pA_2–pA_{10}) difference would be expected to lie on the border-line between competitive and non-competitive types of action. If it were shown that the resolved isomers of promethazine had different types of antagonism, this suggestion would be strongly supported by analogy with the foregoing results for atropine and its stereo-isomers.

Summary

1. Studies of ACh antagonism by hyoscyamine show that (−)-hyoscyamine is 30 times more potent than (+)-hyoscyamine, whereas the potency of the racemic compound, atropine, falls between the values for the stereo-isomers.

2. With atropine and (+)-hyoscyamine the antagonism is non-competitive, whereas antagonism by (−)-hyoscyamine is competitive.

The author wishes to thank the Wellcome Foundation, London, for a supply of (+)-hyoscyamine, and Dr. C. G. Haining, of T. and H. Smith Ltd., Edinburgh, for supplying (−)-hyoscyamine.

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EFFECT OF NICOTINHYDROXAMIC ACID METHIODIDE ON HUMAN PLASMA CHOLINESTERASE INHIBITED BY ORGANOPHOSPHATES CONTAINING A DIALKYLPHOSPHATO GROUP

BY

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(RECEIVED MAY 10, 1955)

Organophosphates which have a dialkylphosphato group $\left(\begin{matrix}O\\ \|\\ -P\\ \end{matrix}\!\!\begin{matrix}OR\\ \diagup\\ \diagdown\\ OR\end{matrix}\right)$ are potent inhibitors of cholinesterase. Studies of the inhibited enzyme indicate that the reaction between cholinesterase and organophosphates of this type yields a phosphorylated enzyme which then reacts with water to produce the corresponding acid and the regenerated enzyme. Reactivation in aqueous solution is fastest if R is a methyl group, but proceeds only very slowly if R is an ethyl group; no measurable enzyme recovery takes place if R is an *iso*propyl group (Burgen and Hobbiger, 1951; Aldridge and Davison, 1953; and Hobbiger, 1954). If the "phosphorylation theory" is correct, then potent nucleophilic reagents should dephosphorylate the enzyme at a greater rate than water. This is indeed so, as was shown by Wilson and Meislich (1953). They found that, after inhibition by an organophosphate with a diethyl- or di*iso*propylphosphato group, the activity of true cholinesterase of electric eel tissue could be completely restored by nicotinhydroxamic acid methiodide (NHA). The rate of enzyme reactivation by 0.1 M-NHA greatly exceeded that produced by water, and at 24° C. 91% of the enzyme activity was restored in 15 min. Wilson and Meislich's results cannot be generally applied because cholinesterases from different sources are not identical in their behaviour towards inhibitors (Austin and Berry, 1953; Davison, 1953). For this reason, and bearing in mind any possible importance in man, the effect of NHA on human plasma cholinesterase, which had been inhibited by organophosphates with a diethyl- or di*iso*propylphosphato group, was studied. The results are presented here.

METHODS

All experiments were carried out in the Warburg apparatus at 37° C. with an atmosphere of 95% N_2 + 5% CO_2. In some experiments 95% O_2 + 5% CO_2 was used and identical results were obtained. A solution containing 0.025 M-$NaHCO_3$, 0.075 M-NaCl, 0.075 M-KCl, 0.04 M-$MgCl_2$ and 0.1% crystalline bovine plasma albumin (Armour Laboratories) was used for all dilutions, and as the medium for inhibition, reactivation, and determination of enzyme activity.

The enzyme preparations were either heparinized human plasma or its highly purified cholinesterase fraction (Plasma fraction IV-6-3 which was a gift from the late Dr. Cohn and had been prepared by the method of Surgenor, Strong, Taylor, Gordon, and Gibson, 1949). Cholinesterase activity was determined by the manometric technique using 0.03 M-butyrylcholine iodide as the substrate. The CO_2 output from 5 to 45 min. after onset of substrate hydrolysis was chosen as a measure of enzyme activity and all reactivations are expressed as a percentage of the activity of controls which had been treated in the same way but without the addition of inhibitor. Dilutions and addition of the reactivating agent were carried out at room temperature, and the moment when the manometric vessels were placed in the bath was chosen as the most accurate, but necessarily arbitrary, zero point of reactivation. Thus the figures obtained for 10 min. contact with the reactivator also include the reactivation which took place at room temperature, and true reaction rates are only represented by the differences between various periods of reactivation.

Solutions of NHA were freshly made immediately before use and adjusted with NaOH to *p*H 7.4 at which all the experiments were carried out.

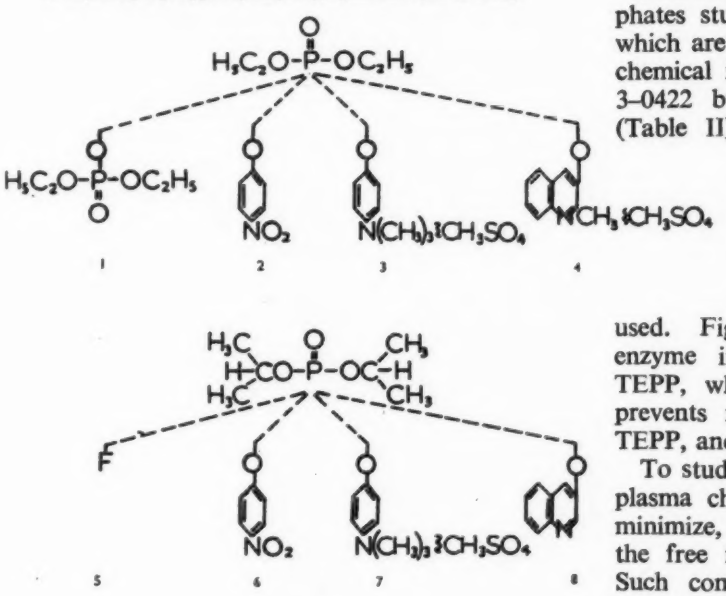
The organophosphates used had either a diethyl- or di*iso*propylphosphato group in common; their structural formulae are shown in Table I. Throughout the text the inhibitors are referred to by the abbreviations given in parentheses in Table I.

TABLE I

ORGANOPHOSPHATES USED AS INHIBITORS

(*a*) Organophosphates containing a diethylphosphato group:
1. Tetraethyl pyrophosphate (TEPP).
2. Diethyl *p*-nitrophenyl phosphate (E 600).
3. 3-(Diethoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3–0340).
4. 3-(Diethoxyphosphinyloxy)-*N*-methylquinolinium methylsulphate (Ro 3–0422).

(*b*) Organophosphates containing a di*iso*propylphosphato group:
5. Di*iso*propyl phosphorofluoridate (DFP).
6. Di*iso*propyl *p*-nitrophenyl phosphate (D 600).
7. 3-(Di*iso*propoxyphosphinyloxy)-*N*-trimethylanilinium methylsulphate (Ro 3–0411).
8. 3-(Di*iso*propoxyphosphinyloxy)-quinoline (Ro 3–0433).



RESULTS

Effect of NHA on Human Plasma Cholinesterase

NHA is a competitive inhibitor of human plasma cholinesterase with an I 50 of 3×10^{-2} M in the presence of 0.03 M-butyrylcholine iodide. The enzyme-inhibitor complex dissociates readily after dilution, and at 37° C. an equilibrium between enzyme, inhibitor and substrate is established within 5 min., regardless of the order in which the 3 components are added. As with other reversible anticholinesterases, NHA partially protects the enzyme against inhibition by organophosphates; but the degree of protection varies according to the type of inhibitor used.

Effect of NHA on Human Plasma Cholinesterase after Inhibition by an Organophosphate with a Diethylphosphato Group

The inhibition of human plasma cholinesterase by TEPP, Ro 3–0340, Ro 3–0422 or E 600 cannot be reversed by addition of substrate, nor after a dilution which is sufficient to reduce the inhibitor concentration to an ineffective level is there any significant enzyme recovery within 1 hr.

The addition of NHA in concentrations above 10^{-3} M to human plasma cholinesterase which has been completely inhibited by one of the four organophosphates markedly increases enzyme activity within 1 hr. The speed and degree of enzyme reactivation at 37° C. and *p*H 7.4 depend on the concentration of free inhibitor, the concentration of NHA, and the time of contact between enzyme and inhibitor.

NHA reacts with each of the four organophosphates studied, and this reaction yields products which are without anticholinesterase activity. The chemical inactivation is rapid with TEPP and Ro 3–0422 but slow with Ro 3–0340 and E 600 (Table II). Aqueous solutions of the organophosphates show the same differences with respect to their stability.

In the presence of free inhibitor the rate of reactivation of inhibited human plasma cholinesterase depends on the nature and the amount of inhibitor used. Fig. 1 shows that NHA reactivates the enzyme in the presence of large amounts of TEPP, whereas a similar excess of Ro 3–0340 prevents reactivation. Ro 3–0422 behaves like TEPP, and E 600 like Ro 3–0340.

To study the true rate of reactivation of human plasma cholinesterase by NHA it is essential to minimize, or if possible exclude, any interference by the free inhibitor with the reactivation process. Such conditions are obtained by incubating a concentrated solution of highly purified human plasma cholinesterase (plasma fraction IV-6-3) with amounts of the inhibitor just sufficient to produce nearly complete inhibition, and then diluting the enzyme-inhibitor mixture a hundredfold before addition of NHA. Using such a procedure it is found that the rate of enzyme reactivation by NHA increases exponentially if enzyme and inhibitor are in contact for not more than 10 min. before reactivation is started. If 20 or 30 min. are allowed for contact between enzyme and inhibitor before the addition of NHA the time-course of

TABLE II

INTERACTION OF NHA AND ORGANOPHOSPHATES AT 37° C.

10^{-3} M and 10^{-7} M solutions of the organophosphates in a phosphate buffer of *p*H 7.4 were incubated with 0.05 M-NHA for 1 hr. The change in anticholinesterase potency was then determined manometrically

Inhibitor	% Reduction in Activity	
	10^{-3} M	10^{-7} M
TEPP	Complete loss of activity	Complete loss of activity
Ro 3–0422		
E 600	0	24
Ro 3–0340	0	19

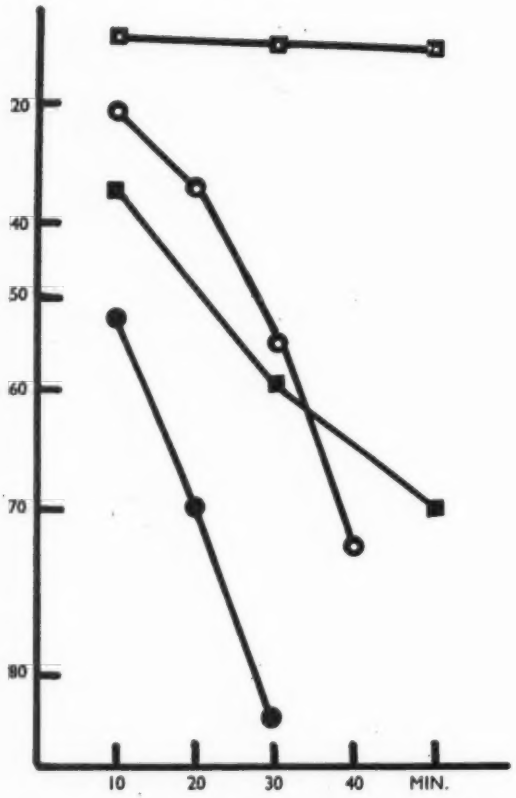


FIG. 1.—Enzyme reactivation by 0.02 M-NHA in presence of excess of free inhibitor. Heparinized human plasma was incubated with the inhibitor for 20 min. before addition of NHA (using 1 vol. NHA for 2 vol. of enzyme-inhibitor mixture). The inhibitors used were: 10^{-8} M-TEPP (●), 10^{-8} M-TEPP (○), 2.5×10^{-8} M-Ro 3–0340 (■) and 2.5×10^{-7} M-Ro 3–0340 (□). Enzyme activity was determined after a 10-fold dilution. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

reactivation approaches that of a first order reaction with one component in excess (Fig. 2). In either of these two cases 0.01 M-NHA restores more than 90% of the activity of a completely inhibited enzyme and an equal degree of reactivation is obtained by a given concentration of NHA regardless of the type of inhibitor used, the variations in different experiments amounting to less than 10%. The time required for maximum reactivation is inversely proportional to the concentration of NHA (Fig. 2).

These findings indicate that under the experimental conditions NHA acts on a phosphorylated enzyme. This interpretation is supported by the effect of trimethylamine on enzyme reactivation by NHA. Wilson and Meislich (1953) have shown that NHA is better than hydroxylamine as a reactivating agent for cholinesterase of electric eel tissue; the most plausible explanation for this is that the quaternary nitrogen group of NHA becomes attached to the anionic site of the enzyme. The presence of an anionic site in human plasma cholinesterase has been demonstrated by Bergmann and Wurzei (1954). If organophosphates do phosphorylate human plasma cholinesterase a block of the anionic site by trimethylamine should slow down enzyme reactivation by NHA. This is indeed so no matter which of the four organophosphates is used as the inhibitor and in spite of the fact that Ro 3–0340 and Ro 3–0422 also possess a quaternary nitrogen group which occupies the anionic site of the enzyme as long as the inhibitor molecule remains intact (Table III). Butyrylcholine—the substrate used throughout the experiments—has the same effect as trimethylamine.

If organophosphates with a diethylphosphato group are used for *in vivo* work complete enzyme recovery requires several days. Experiments were, therefore, carried out to study the effect of NHA on human plasma cholinesterase which had been incubated with the inhibitor for long periods. These experiments showed that the reactivating power of NHA decreases gradually as the time of contact between enzyme and inhibitor is increased (Fig. 3). The findings are identical with all four inhibitors and are not due to a gradual deterioration

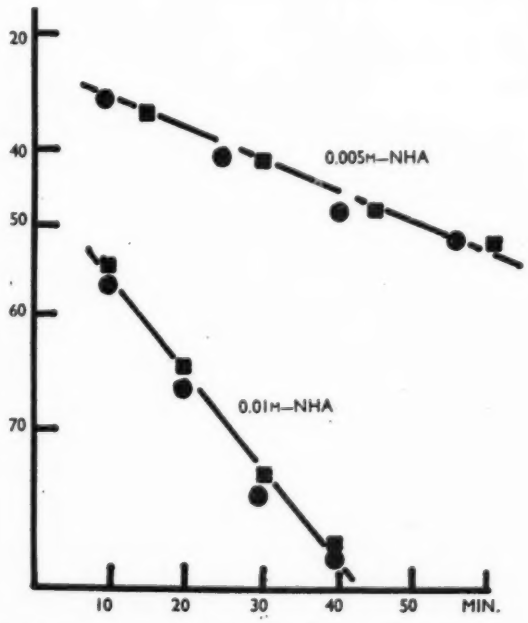


FIG. 2.—Enzyme reactivation by NHA without interference by free inhibitor. A solution of purified human plasma cholinesterase was incubated with 5×10^{-8} M-TEPP (●) or 10^{-7} M-E 600 (■) for 30 min. and then diluted 100-fold with NHA. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

TABLE III

EFFECT OF TRIMETHYLAMINE ON ENZYME REACTIVATION BY NHA

NHA or NHA and trimethylamine were added to purified plasma cholinesterase which had previously been incubated with 5×10^{-8} M Ro 3–0340 for 20 min. and was then diluted 10-fold. The activity of individual samples was determined after a 10-fold dilution with substrate

	Activity in % of Control after Reactivation for		
	10 min.	30 min.	50 min.
(*a*) Inhibited enzyme alone ..	3	2	2
(*b*) (*a*) + 0·05 M-NHA	74	95	98
(*c*) (*a*) + 0·05 M-NHA + 0·7 M trimethylamine	25	49	63

of the enzyme during the prolonged incubation at 37° C. Enzyme solutions which were kept for 24 hr. at 37° C. and then incubated with the inhibitor for 30 min. behaved like freshly made enzyme solutions which had been inhibited for the same time.

Effect of NHA on Human Plasma Cholinesterase after Inhibition by an Organophosphate with a Diisopropylphosphato Group

The inhibition of human plasma cholinesterase by DFP, D 600, Ro 3–0411 or Ro 3–0433—like that produced by organophosphates with a diethylphosphato group—can neither be reversed by substrate nor is there any measurable enzyme recovery within a few hours after a dilution which is sufficient to reduce the inhibitor concentration to ineffective levels.

The addition of NHA to dilute solutions of any one of the organophosphates leads to a loss of the anticholinesterase potency of the inhibitor. The rate of interaction of the inhibitor with NHA is of the order DFP>D 600>Ro 3–0433>Ro 3–0411 and is thus a reflection of the stability of the inhibitor in aqueous solution (Table IV).

TABLE IV

STABILITY OF ORGANOPHOSPHATES WITH A DI*ISO*-PROPYLPHOSPHATO GROUP AND THEIR RATE OF INTERACTION WITH NHA

A: Loss of activity of 10^{-5} M solutions which were kept for 2 hr. at 80° C. B: Loss of activity of 10^{-5} M solutions which were kept with 0.01 M-NHA for 2 hr. at 37° C. All incubations were carried out in a phosphate buffer of *p*H 7.4

Inhibitor	% Reduction in Activity	
	A	B
DFP	99·9	72
D 600	54	13
Ro 3–0411	33	5
Ro 3–0433	39	8

If human plasma cholinesterase is incubated with DFP, D 600, Ro 3–0411 or Ro 3–0433, NHA can restore some of the enzyme activity. The degree of enzyme reactivation at 37° C. and *p*H 7.4 depends on the time of contact between enzyme and inhibitor, the concentration of NHA, and the type of inhibitor used.

The presence of free inhibitor during the incubation of inhibited enzyme with NHA interferes with the reactivation process in the same way as was shown previously for the reactivation of enzyme inhibited by an organophosphate with a diethylphosphato group.

The results obtained with NHA fall into two distinct groups according to which organophosphate is used as the inhibitor:

(1) *Inhibition by DFP or D* 600.—If human plasma cholinesterase is inhibited by DFP or D 600 NHA never completely restores enzyme activity. The degree of enzyme reactivation by NHA is inversely proportional to the time of contact between enzyme and inhibitor and approaches a maximum within 10 min. (Figs. 4 and 5). If the enzyme is incubated with the inhibitor for more than 1 hr. no enzyme reactivation is obtained with 0.1 M-NHA. Trimethylamine interferes with the action of NHA, but its effect is less than after inhibition by an organophosphate with a diethylphosphato group.

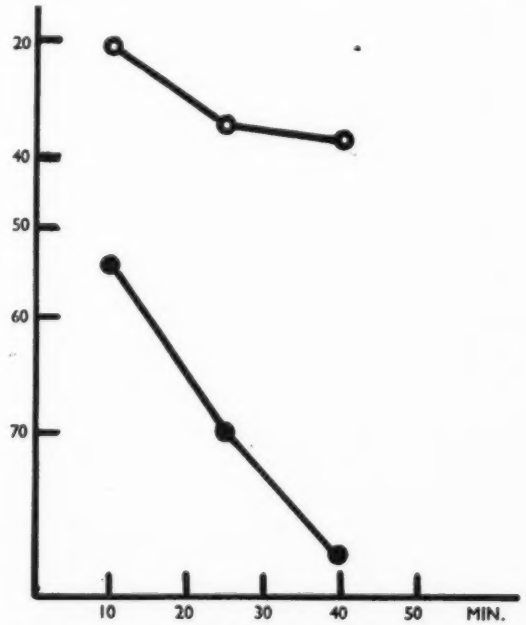


FIG. 3.—Enzyme reactivation by 0.01 M-NHA after various times of contact between enzyme and inhibitor. Purified human plasma cholinesterase was incubated with 5×10^{-8} M-E 600 for 30 min. (●) and 24 hours (○). The enzyme-inhibitor mixture was then diluted 100-fold with NHA. Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

(2) *Inhibition by Ro* 3–0411 *or Ro* 3–0433.—If NHA is added to human plasma cholinesterase which has been inhibited by Ro 3–0411 or Ro 3–0433 enzyme reactivation is always more marked than if the enzyme had been incubated with DFP or D 600 for the same time (Fig. 4). The degree of enzyme reactivation by NHA is inversely related to the duration of inhibition, but a considerable amount of enzyme activity can still be restored by 0.1 M-NHA even after 1 hr. of contact between enzyme and inhibitor (Fig. 5). Reactivation by NHA proceeds progressively, but its rate falls considerably short of that of a first order reaction.

The differences in the effect of NHA on enzyme inhibited by organophosphates of groups (1) and (2) are not due to interference by hydrolysis products which arise during phosphorylation of the enzyme.

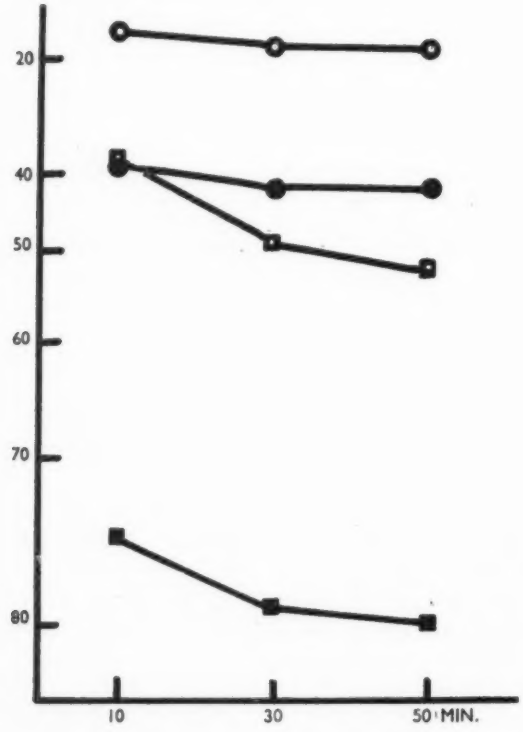


FIG. 4.—Effect of NHA on enzyme activity after inhibition by DFP or Ro 3–0433. Purified plasma cholinesterase was incubated for 10 min. with 5×10^{-8} M-DFP or 2×10^{-7} M-Ro 3–0433. Part of the enzyme-inhibitor mixture was then diluted 10-fold with NHA (final concn.: 0.1 M) and reactivation allowed to proceed for 10, 30, and 50 min. Enzyme activity was determined after a further 10-fold dilution. (●: enzyme inhibited by DFP; ■: enzyme inh bited by Ro 3–0433). The remainder of the enzyme-inhibitor mixture was diluted 100-fold with NHA (final concn.: 0.01 M) and reactivation determined after 10, 30, and 50 min. (○: enzyme inhibited by DFP; □: enzyme inhibited by Ro 3–0433). Ordinate: enzyme activity in % of controls. Abscissa: duration of exposure of inhibited enzyme to NHA before addition of substrate.

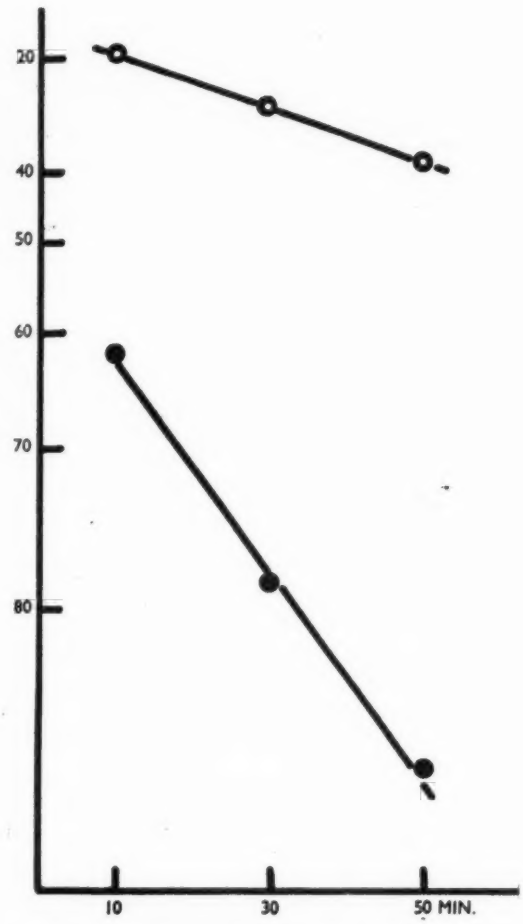


FIG. 5.—Amount of enzyme activity which cannot be restored by 0.1 M-NHA after incubation of plasma cholinesterase with 5×10^{-8} M-DFP (●) or 2×10^{-7} M-Ro 3–0433 (○). Purified plasma cholinesterase was incubated with the inhibitor for 10, 30, and 50 min. The enzyme-inhibitor mixtures were then diluted 10-fold with NHA (final concn.: 0.1 M). 40 min. were allowed for reactivation and enzyme activity was then determined after a further 10-fold dilution. Abscissa: time of contact between inhibitor and enzyme before addition of NHA. Ordinate: % of enzyme activity which cannot be restored by 0.1 M-NHA.

TABLE V

REACTIVATION OF HUMAN PLASMA CHOLINESTERASE INHIBITED BY TWO DIFFERENT ORGANOPHOSPHATES

Purified human plasma cholinesterase was incubated for 10 min. with either 10^{-7} M-DFP (*a*) or 2×10^{-7} M-Ro 3–0433 (*b*). Equal parts of *a* and *b* were then mixed (*c*) and all three samples incubated for another 50 min.; *a*, *b*, and *c* were then diluted 10-fold with NHA (final concentration 0.1 M) and 40 min. allowed for reactivation which was determined on equal volumes of *a*, *b*, and *c* after a further 10-fold dilution

	Observed Reactivation in % of Control	Calculated Reactivation in % of Control
a	2	—
b	53	—
c	28	27·5

Table V shows that the effect of NHA on human plasma cholinesterase which, after inhibition by DFP, was further incubated with another sample of the same enzyme previously inhibited by Ro 3–0433 is exactly the same as would be expected from the effect of NHA on two control samples of the enzyme which had been independently inhibited.

Discussion

Human plasma cholinesterase which has been inhibited by an organophosphate with a diethyl- or di*iso*propylphosphato group can be reactivated by NHA. The degree of enzyme reactivation which is obtained with NHA at 37° C. and *p*H 7.4 depends on the time of contact between enzyme and inhibitor, the concentration of NHA, and the type of inhibitor.

With organophosphates which contain a diethylphosphato group (TEPP, E 600, Ro 3–0340 and Ro 3–0422) the findings indicate that the inhibitory process involves the following stages:

> Enzyme + inhibitor⟶addition complex⟶phosphorylated enzyme I⟶phosphorylated enzyme II.

All four organophosphates react with NHA, and this reaction yields products which are without anticholinesterase activity. The chemical inactivation of TEPP and Ro 3–0422 is much faster than that of Ro 3–0340 and E 600, but reactivation of the inhibited enzyme by NHA (in the absence of free inhibitor) is the same regardless of which inhibitor is used. Such results are only possible if NHA is acting on a phosphorylated enzyme. This interpretation is fully supported by the finding that trimethylamine, by blocking the anionic site of the enzyme, slows the rate of enzyme reactivation.

The degree of enzyme reactivation obtained with NHA is inversely related to the duration of inhibition. If the enzyme has been in contact with the inhibitor for up to 30 min., more than 90% of its activity is restored. After 24 hr. contact with the inhibitor, NHA restores less than 50% of the activity. This must mean that the diethylphosphato group of the inhibitor after its initial attachment to one reactive group of the esteratic site (phosphorylated enzyme I) is slowly transferred to another reactive group with which it forms a bond of higher stability and unable to react with NHA (phosphorylated enzyme II).

The inhibitory reaction of human plasma cholinesterase with organophosphates containing a di*iso*propylphosphato group is also a phosphorylation process which leads to the formation of two different types of phosphorylated enzyme. After equal periods of contact between enzyme and inhibitor, NHA always produced a greater reversal of the inhibition by Ro 3–0411 or Ro 3–0433 than it did of the inhibition by DFP or D 600. The rate of interaction between NHA and the four organophosphates is of the order DFP>D 600>Ro 3–0433>Ro 3–0411, so that if the reactivation obtained by adding NHA to the inhibited enzyme were due to an interaction between NHA and the intact inhibitor molecule—as it would be with an addition complex—the inhibition produced by DFP should be more easily reversed by NHA than that produced by the other inhibitors. The experimental findings show the reverse. Since there is no interference by the products which arise during hydrolysis of the organophosphates it must be assumed that Ro 3–0411 and Ro 3–0433 transfer their di*iso*propylphosphato groups to a reactive group of the esteratic site which is different from that to which the di*iso*propylphosphato group of DFP and D 600 is initially attached. If this is so it is not surprising that the rate of formation of a phosphorylated enzyme which cannot be reactivated by NHA (phosphorylated enzyme II) is different with different inhibitors. With DFP and D 600 all the inhibited enzyme is present in form of phosphorylated enzyme II if the time of contact between enzyme and inhibitor exceeds 1 hr. With Ro 3–0411 and Ro 3–0433 the formation of a stable bond between the enzyme and the di*iso*propylphosphato group is slower, but it is still considerably faster than with inhibition by organophosphates containing a diethylphosphato group.

It is possible that the findings reported here are not entirely applicable to *in vivo* conditions and that the rate of formation *in vivo* of a phosphorylated enzyme which cannot be reactivated by NHA (phosphorylated enzyme II) differs greatly. That two types of phosphorylated enzyme are formed *in vivo* is, however, indicated by the results of Davison (1953), who found that the true cholinesterase of rats with E 600 poisoning recovers 50 to 60% of its activity within 4 days, whereas the later stage of recovery proceeds at a considerably slower rate.

Wilson and Meislich (1953), who first described the use of NHA as a reactivator of phosphorylated cholinesterase, found that at 24° C. reactivation of an enzyme inhibited by DFP increased progressively during a period of 24 hr. The differences in the enzymes—human plasma cholinesterase instead of electric eel cholinesterase—and in the temperature —37° C. instead of 24° C.—are most probably the reasons for the lack of agreement between Wilson and Meislich's findings and the results reported here.

In recent years attempts have been made to study the active surface of carboxylic esterases using ^{32}P

labelled DFP as an inhibitor of chymotrypsin, bovine true cholinesterase, human plasma cholinesterase and the aliphatic esterase of red cell stromata. If the inhibited enzymes are subjected to proteolytic digestion followed by treatment with concentrated HCl, serine phosphate containing the labelled P atom can be isolated (Schaffer, May and Summerson, 1953; Cohen, Osterbaan and Warringa, 1954; and Osterbaan, Kunst and Cohen, 1955). Should the findings which are reported here also apply to other carboxylic esterases it would appear that other amino-acids, in addition to serine, participate in the inhibitory reaction, and methods of analysis will have to be found for detecting those parts of the enzyme which play an important role in those initial stages of the inhibition which lead to the formation of phosphorylated enzyme I.

Preliminary experiments have shown that the results with human plasma cholinesterase also apply to the true cholinesterase of human red cells. With TEPP as inhibitor, NHA is about 20 times less potent as a reactivating agent of true cholinesterase than of plasma cholinesterase; thus no great beneficial result could be expected from its use in human beings with organophosphate poisoning.

Summary

1. The effects of nicotinhydroxamic acid methiodide (NHA) on human plasma cholinesterase inhibited by organophosphates containing either a diethyl- or a di*iso*propylphosphato group were studied.

2. The inhibition produced by four different organophosphates with a diethylphosphato group (TEPP, E 600, Ro 3–0340 and Ro 3–0422) is reversed by NHA. The degree of reactivation depends on the concentration of free inhibitor, the time of contact between enzyme and inhibitor, and the concentration of NHA. The inhibitory reaction is a phosphorylation process producing 2 types of phosphorylated enzyme. Phosphorylated enzyme I, which is formed initially, can be fully reactivated by NHA. Phosphorylated enzyme II is formed during prolonged contact between enzyme and organophosphate and cannot be reactivated by NHA.

3. The inhibition produced by organophosphates with a di*iso*propylphosphato group also leads to the formation of two types of phosphorylated enzyme. With DFP and D 600 as inhibitors reactivation of the enzyme is obtained only if the time of contact between enzyme and inhibitor does not exceed 1 hr., whereas the inhibition produced by Ro 3–0411 and Ro 3–0433 can be partially reversed by NHA even after considerably longer inhibition.

4. The results are discussed in relation to the inhibitory mechanism and the study of the active surface of carboxylic esterases.

I wish to thank Dr. A. L. Morrison, Director of the Research Department of Roche Products, Ltd., for helpful discussions and a generous supply of all the Ro compounds used in this work. I also wish to express my thanks to Dr. R. F. Long, of Roche Products, Ltd., for the synthesis of nicotinhydroxamic acid methiodide.

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THE MECHANISM OF AFTER-DISCHARGES CAUSED BY VERATRINE IN FROG'S SKELETAL MUSCLES

BY

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(RECEIVED MAY 12, 1955)

The veratrine alkaloids seem to affect a wide range of electrically excitable cells in the same way (Krayer and Acheson, 1946). A single stimulus, which in normal circumstances causes a single response, will in the presence of veratrine give rise to a prolonged after-discharge or series of repeated action potentials. The production of after-discharges by the veratrum alkaloids has been observed in peripheral nerve (Feng, 1941), in skeletal muscle (Kuffler, 1945) and in conducting tissue of mammalian ventricle (Goldenberg and Rothberger, 1936). To produce an after-discharge it is not necessary that the whole length of an electrically excitable cell should be bathed in veratrine solution; local application of the alkaloid followed by excitation of any portion of the cell's membrane is sufficient to produce a train of action potentials in response to a single stimulus (Wible, 1924). In skeletal nerve-muscle preparations, it appears that the regions most sensitive to the drug's action are the terminal branches of the motor nerve and the muscle fibres. The frog's sartorius nerve-muscle preparation will give repetitive responses to single motor nerve volleys in the presence of a veratrine concentration of approximately 10^{-7}. The frog's sciatic nerve trunk, however, requires soaking for one hour in a concentration of 10^{-5} veratrine before after-discharges can be produced reliably (Feng, 1941). The action of the drug at motor-nerve terminals does not seem to be due to interference with the normal mechanisms of humoral transmission. Moreover, the muscle fibre itself seems to be equally sensitive to the drug in all regions down its length (Kuffler, 1945). In skeletal muscle, as in peripheral nerve (Graham and Gasser, 1931), veratrine seems to cause a delay in repolarization following an action potential. It is during this process of delayed repolarization following the first driven action potential that the repetitive after-discharge occurs. Kuffler (1945) concluded that this maintained, partial depolarization of the muscle fibre was directly responsible for a state of membrane instability which could give rise to repeated action potentials. He says, "All the evidence available indicates that the effects of the negative after-potentials are analogous to those of cathodal currents applied artificially to nerve or muscle fibres."

Recent experiments with the cat's cerebral cortex (Burns, 1954, 1955) have shown that there is a system of cells in the cortical grey-matter which will normally give a prolonged series of after-discharges following a few driven responses. The experimental results lead to the conclusion that this cortical after-discharge occurred because the different parts of the cells involved did not repolarize at the same rate following their driven activity. It was postulated that after an action potential one end of the type-B neurones (Burns and Grafstein, 1952) repolarized some ten times more slowly than did the other. During this *differential repolarization*, current must flow from the more rapidly repolarizing end of the cells to the other end and this current may be sufficient to cause the development of an action potential. Following the first "spontaneous" or undriven action potential the recovery process is set back a step and the same cycle of events may be repeated, thus giving rise to a series of after-discharges. It was pointed out (Burns, 1955) that the mechanism of differential repolarization might play some part in causing the after-discharges produced by veratrine. This interpretation does not differ seriously from that proposed by Kuffler and quoted above, but would specify the analogy between the negative after-potentials due to veratrine and the effects of applied cathodal currents a little more precisely. Forced uniform depolarization of the cell's surface may lead to an instability of membrane potential and consequent development of a stationary "membrane action potential" (Hodgkin and Huxley, 1952). Alternatively, a local cathode may give rise to transmitted action potentials because it depolarizes

one section of cell membrane *relative* to neighbouring membrane beyond the reach of the polarizing current. In this case the final breakdown of membrane potential would be associated with current flow between normal and cathodally polarized membrane.

Both the mechanisms for after-discharge from veratrinized cells presuppose a persistent depolarization of the membrane, but one supposes that universal, partial depolarization is a sufficient cause for after-discharge, while the other postulates that there must be a depolarization gradient (or differential repolarization) down the length of the cell before repetitive action potentials can occur. We undertook the experiments described below in order to find the extent to which each of these two postulated mechanisms contributed to the formation of the after-discharge. It will be realized that if differential repolarization is the major cause of repetitive response, then the local application of veratrine should be far more effective in producing after-discharge than its universal application to the whole cell. Most of the techniques developed have therefore been concerned with making this comparison.

METHODS

The Preparation.—The sartorius muscle of the frog was used in these experiments. The muscle was first dissected free from the animal and then pinned out on a board. Small strips were cut from the muscle under a dissecting microscope so that the cuts ran parallel to the muscle fibres and from end to end. A strip usually contained some 50 muscle fibres and was about 1 mm. in cross-sectional diameter. Fine silk thread was tied around each end of the strip of muscle and the preparation was lifted into the slot of the saline baths, with its distal end in bath 1 (Fig. 1). In order to insulate the baths from one another electrically, and to prevent interchange of fluid, vaseline was packed around the muscle in the groove joining the baths.

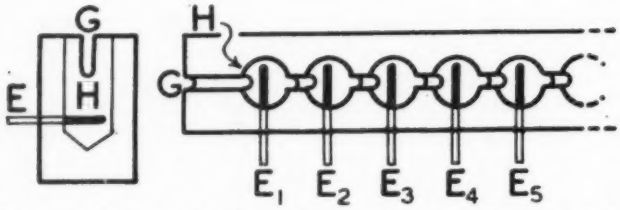

FIG. 1.—A diagram of the perspex bath used for mounting strips of the frog's sartorius muscle. For description see text.

The Saline Bath.—The muscle bath was constructed from perspex (lucite) in such a way that we could either apply drugs to one section of the length of muscle fibres or apply them universally to the whole of the preparation (Fig. 1). Nine holes (H) of 6 mm. diameter were drilled in a perspex block to a depth of 13 mm. The centres of the holes lay in a straight line and were 6.5 mm. apart. The upper one-third of these holes communicated with one another through a milled groove (G) of width 1.5 mm. and depth 4 mm. which ran the whole length of the perspex block. Each hole contained at the lower end a chlorided silver electrode (E) which had been pressed into the perspex block so as to form a watertight seal.

Solutions.—The composition of the Ringer solution used was: NaCl, 6.5 g., $NaHCO_3$, 0.2 g., KCl, 0.14 g., $NaH_2PO_4.H_2O$, 0.012 g., $CaCl_2$, 0.12 g., glucose, 2.0 g., dissolved in 1 litre of glass-distilled water. To the solution was added (+)-tubocurarine chloride obtained from Burroughs Wellcome and Co. Tests were made in the summer of the strength of tubocurarine solution necessary to produce complete neuromuscular block, and we found that a concentration of 10^{-5} was sufficient. Later in the year 10^{-4} was needed in some frogs. In order to demonstrate that the application of veratrine solutions to the whole length of muscle fibres does not produce after-discharges, it is necessary to have sufficient tubocurarine present to reduce the end-plate potentials to very small values. When the tubocurarine concentration is only just sufficient to prevent normal neuromuscular transmission in all fibres, large end-plate potentials may still occur. A stimulus which is intended for direct excitation of muscle fibres will still excite a few intramuscular nerve-twigs; the repetitive response of the latter in the presence of veratrine can give summation of end-plate potentials until a repeating neuromuscular transmission is established (*cf.* Kuffler, 1945). In the early experiments we used Ringer solution to which 10^{-4} (+)-tubocurarine HCl had been added, but for safety we would recommend the use of 10^{-3} tubocurarine, which was employed during the latter part of the work reported below.

Veratrine sulphate (obtained from Brickman & Co., Montreal) was made up as a 10^{-3} stock solution in tubocurarine-Ringer fluid. It will maintain its potency for about three weeks if stored in a refrigerator.

Recording System and Figures.—Most of the records made employed the silver-silver-chloride electrodes fixed in the base of the baths. Occasionally, however, we used a microelectrode filled with soft solder (Burns and Grafstein, 1952) for recording the repetitive activity from one or a few muscle fibres. Potentials from the electrodes were fed through cathode followers to two channels of amplification with coupling time-constants which could be varied from 1 msec. to infinity (=direct coupled). Records were made on film with a Cossor 1049 oscilloscope.

Voltage calibration for most of the records is not given since such calibrations had no particular physiological meaning. The magnitude of the potentials recorded was more dependent upon the perfection of

the vaseline seals than any other factors. A diagram of the recording arrangement is provided with each of the figures. Because in many of the experiments there was a great difference in the oscillograph spot velocities in the X and Y directions, many of the "spikes" have been touched up with white ink in order to make the figures clearer.

RESULTS

A Comparison Between Local and General Application of Veratrine

In most of our preparations a few muscle fibres began to give a repetitive response after local exposure to 10^{-7} veratrine for 5 min. The veratrine solution was usually applied in two adjacent baths (bath 1 and 2 of Fig. 1) of the seven baths through which the muscle passed; the threshold concentration for repetitive response to a single stimulus (given to the untreated portion of the muscle) varied from preparation to preparation but always lay between 0.5×10^{-7} and 0.5×10^{-6}. Concentrations of veratrine greater than 10^{-6} are liable to block transmission. Once the required threshold concentration for the local application of the drug had been found, a repetitive response to single stimuli could be maintained indefinitely. Test stimuli given once every half minute or minute would elicit the after-discharge in repeatable fashion for more than 5 hr. Occasionally, the repetitive response would disappear after about half an hour of testing, but we were always able to show that on these occasions the veratrine had leaked from the baths in which it was applied into a neighbouring bath. As a precaution against this failure to localize the action of the drug, we usually perfused the bath next to that which contained the veratrine solution (usually bath 3 of Fig. 1) with a slow flow of tubocurarine-Ringer solution.

When veratrine was supplied to baths 1 and 2 but the rest of the muscle was bathed with tubocurarine-Ringer solution, there was a great difference in the repolarization rates of the veratrinized and untreated lengths of muscle fibre, following a driven action potential. Records made from leads in baths 2 and 3 (with the amplifier direct coupled) showed a long-lasting relative negativity of bath 2, upon which the activity of repeating muscle fibres was superimposed (Fig. 5). Leakage of the veratrine from bath 2 into bath 3 caused an immediate decrease of this difference in repolarization rates, and usually produced a reduction in the after-discharge (see Fig. 2).

In contrast to the efficiency of the local application of veratrine in producing after-discharge, the

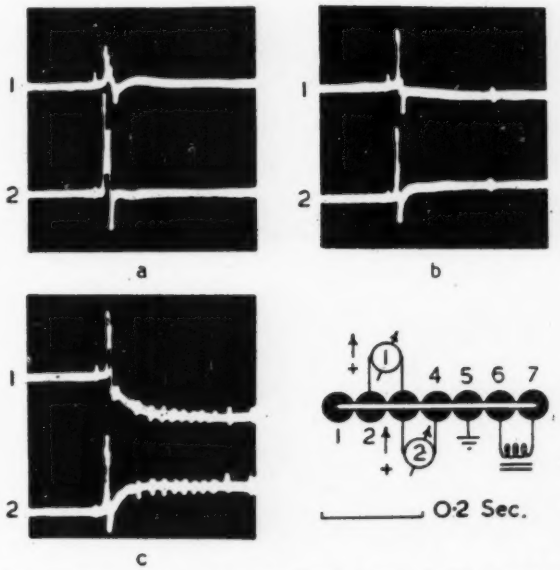

FIG. 2.—The responses to a single stimulus of the sartorius muscle to general and to restricted application of veratrine. (*a*) After immersion in 10^{-7} veratrine sulphate for 80 min. (*b*) 20 min. after beginning of continuous perfusion of bath 3 with tubocurarine-Ringer solution, in order to wash out the veratrine. (*c*) 130 min. later.

application of the veratrine in the same concentration to all the baths through which the muscle passed caused no repetition following a single stimulus. Soon after the application of veratrine to the whole length of the muscle fibre there was usually an increase in after-discharge, but within a few minutes the muscle fibres reverted to a state in which a single stimulus produced a single response, and stayed in this condition indefinitely. The results of Fig. 2, which are from a modification of this experiment, illustrate this point. Fig. 2a shows the response to stimulation of a strip of muscle which had been soaked in 10^{-7} veratrine solution for 80 min. before it was placed in the series of baths, all of which contained veratrine in the same concentration. The single stimulus produced no after-discharge. We then began washing out the veratrine in bath 3 by perfusion with tubocurarine-Ringer solution; Fig. 2b shows a single after-discharge occurring probably in one muscle fibre of the bundle, 20 min. after washing was begun. Fig. 2c shows the after-discharge recorded after bath 3 had been washed through for 2½ hr. Fig. 2b and c also shows the development of a more rapid rate of repolarization for the muscle in bath 3 as the veratrine is washed out. For example, in Fig. 2 the driven action potential is passing from right to left along the muscle fibre, and channel I records its passage through bath 3 and 2 as an upward, followed by a downward,

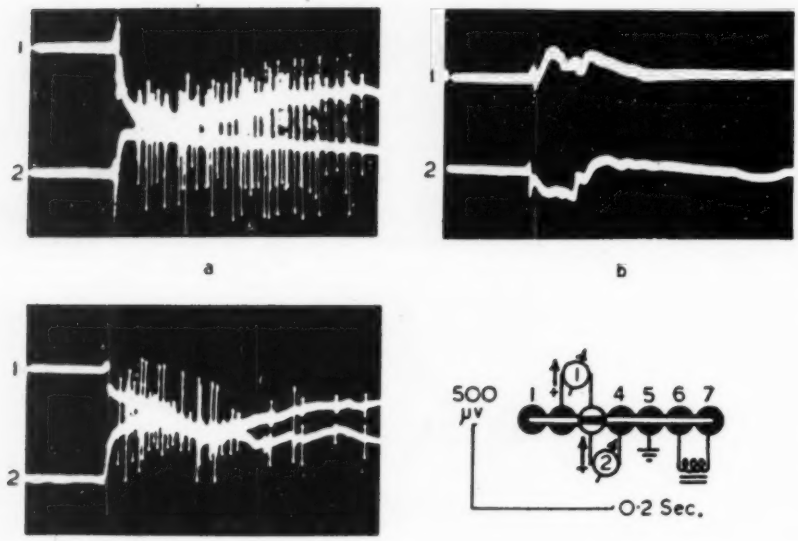

FIG. 3.—The responses to a single stimulus of the sartorius muscle to general and restricted application of veratrine. (*a*) 0.25×10^{-6} veratrine sulphate in all baths except 3. (*b*) 47 min. after 0.25×10^{-6} veratrine sulphate added to bath 3. (*c*) 70 min. after beginning continuous perfusion of bath 3 with tubocurarine-Ringer solution in order to wash out the veratrine.

deflection. The downward deflection implies that the muscle in bath 2 is depolarized at a time when that in bath 3 is recovered; any prolongation of this downward deflection (as in Fig. 2b or c, channel I) indicates that the muscle in bath 2 must be repolarizing much more slowly than that in bath 3.

Essentially the same result is obtained when the muscle is exposed to the same conditions in the reverse order. A reliable after-discharge in response to a single stimulus can be obtained when all of the baths, through which the sartorius strip passes, contain veratrine except one. The repetitive response shown in Fig. 3a was obtained as a repeatable phenomenon, when baths 1, 2, 4, 5, 6, and 7 contained 0.25×10^{-6} veratrine, although bath 3 contained tubocurarine-Ringer solution. The diphasic records make it clear that muscle fibres treated with this comparatively low concentration of veratrine can transmit a series of action potentials at high frequency. Soon after bath 3 had been filled with veratrine of concentration 0.25×10^{-6} so that the whole muscle was submerged in a uniform veratrine concentration, the after-discharge to the test stimulus disappeared (Fig. 3b). Finally, when the veratrine in bath 3 was washed out with Ringer solution, the after-discharge returned (Fig. 3c).

In the experiments described so far, the veratrine solution was either applied locally to one stretch of muscle fibre or was applied to the whole fibre. In other words, either there was a maintained concentration gradient of the drug down the length of the fibres or the concentration gradient was made zero. If the necessary condition for after-discharge is a sufficient concentration gradient, it should be possible to stop the after-discharges which occur when a threshold quantity of veratrine is in only one bath, by the addition of a *subthreshold quantity* of veratrine to the neighbouring baths. In the experiment whose results are given in Fig. 4, 0.5×10^{-7} veratrine was first added to baths 1 and 2. This concentration of veratrine was only just above the threshold for after-discharge of the fibres whose activity is shown in Fig. 4a and b. When 0.25×10^{-7} veratrine was added to bath 3 the repeated response to a single test stimulus soon disappeared (Fig. 4c and d), and only returned again when the veratrine in bath 3 was washed out (Fig. 4e and f). The experiment

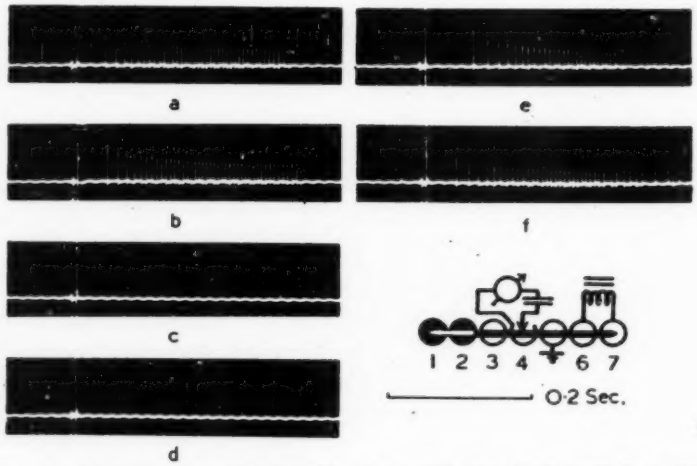

FIG. 4.—Responses to a single stimulus of a muscle fibre in the sartorius to local application of veratrine sulphate, recorded from a metal micro-electrode in bath 4. (*a*) 0.5×10^{-7} veratrine sulphate in baths 1 and 2 only (for 34 min.). (*b*) Same as (*a*) 4 min. later. (*c*) 2 min. after beginning of perfusion of bath 3 with 0.25×10^{-7} veratrine sulphate. (*d*) 3 min. later. (*e*) 6 min. after beginning perfusion of bath 3 with tubocurarine-Ringer solution in order to wash out veratrine. (*f*) 13 min. later.

of Fig. 4 required considerable patience during the initial administration of veratrine to baths 1 and 2 because each addition of the drug took some 15 min. to reach its full effect under our conditions of experiment. Moreover, it will be seen that the results of Fig. 4 can only be obtained if the concentration in these first two baths is only a little above threshold for the after-discharge. Only then can the subsequent concentration gradients between baths 2 and 3 and between baths 3 and 4 both be below threshold for after-discharge.

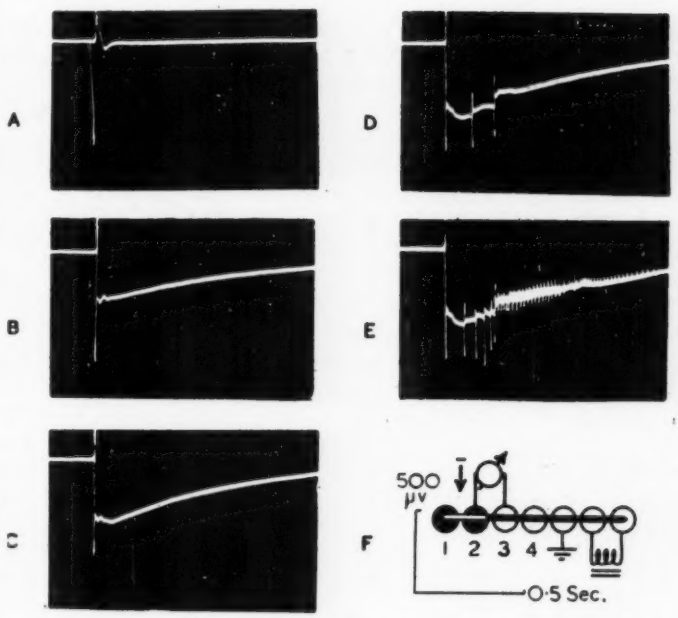

FIG. 5.—Responses to a single stimulus in a small bundle of fibres from the sartorius muscle during the progressive local application of veratrine sulphate. (*a*) Control. Whole of muscle in tubocurarine-Ringer solution. (*b*) 34 min. after 0.75×10^{-7} veratrine sulphate in baths 1 and 2. (*c*) 12 min. later. After a further 6 min. 10^{-7} veratrine sulphate put in baths 1 and 2. (*d*) 7 min later than previous record. (*e*) 1 min. later.

The Mechanism of Action of a Veratrine Concentration Gradient

The experiments described above make it clear that frog's skeletal muscle, physiologically isolated from its nerve supply, will only maintain a repetitive response to direct excitation if veratrine is locally applied, and not if it is uniformly applied over the length of the muscle fibres. It is presumably the well-known delayed repolarization of the veratrinized length of muscle which is responsible for this after-discharge. After the driven action potential has swept through the normal and veratrinized muscle, the latter repolarizes much more slowly than does the former (see Fig. 5b). The different rates of repolarization must cause current to flow in the extracellular spaces from normal to veratrine-treated muscle, and it is this current flow at the boundary of veratrinized and normal muscle which presumably causes the origin of an action potential. This concept implies that the threshold concentration of veratrine necessary to produce an after-discharge should be accompanied by a measurable threshold difference of membrane potential between normal and treated membrane. Fig. 5b, c, d, and e shows the development of such a potential difference as a concentration of 10^{-7} veratrine slowly attains its full effect. In Fig. 5b and c the potential difference during recovery from the driven action potential between veratrinized and normal muscle (baths 2 and 3) is not sufficient to trigger an after-discharge. Seven min. later this potential difference is adequate for after-discharge (Fig. 5d and e). The repetitive response recorded with a high velocity sweep from baths 2 and 3 showed that each action potential was diphasic with the first phase down. This indicated that the after-discharge originated in the neighbourhood of the treated stretch of muscle and its activity was therefore travelling in the opposite direction to that of the driven action potential. In Fig. 6 is shown the consequence of two driven action potentials on a similar preparation. The potential

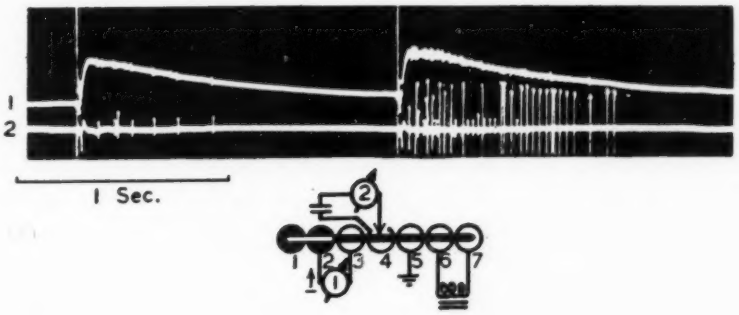

FIG. 6.—The responses of a small bundle of muscle fibres to two stimuli in the presence of local veratrine. Channel 1 shows a D.C. record from baths 2 and 3. Channel 2 records the activity of a few fibres with a metal microelectrode in bath 4. 10^{-7} veratrine sulphate in baths 1 and 2 only.

gradient between normal and veratrinized muscle is only sufficient to trigger a repetitive response from two fibres during recovery from the first volley, but the after-effects of the second volley add to those of the first so that the potential gradient is finally brought above threshold for after-discharge of four or five fibres.

Another way in which the dependence of after-discharge upon a suprathreshold potential gradient can be shown is by varying the frequency of the driving stimulus. A local concentration of veratrine which is sufficient to cause a repetitive response to stimuli given at one a minute may never cause after-discharge if the driving stimuli occur at two per minute. The magnitude of the after-potential (or delayed phase of repolarization) which follows a driven action potential increases measurably with increase of the rest allowed up to periods of about 2 min. As would be suspected, the probability of a stimulus causing an after-discharge is also increased by prolonging the rest which precedes the triggering stimulus.

Experiments with Polarizing Current

The experiments described so far make it clear that the veratrine after-discharges will only occur provided that during recovery from a driven action potential a sufficient gradient of membrane potential per unit length of fibre exists. For any one muscle fibre the value of this potential gradient will depend only upon the magnitude of the peak local concentration of veratrine, and upon the concentration gradient of veratrine down the length of the fibre.

This concept of the action of local veratrine application implies that one should be able to modify the veratrine after-discharge with polarizing currents applied to that part of the veratrinized muscle which lies close to the untreated length of the fibres. Polarization was effected by switching on a measured current, which entered one bath of the series in which the muscle lay, and left by another bath ; the current entered and left these baths through non-polarizable electrodes separate from those used for recording purposes. When the preparation is treated with local veratrine, the addition of a cathode in the veratrinized bath (No. 2) will increase the veratrine after-discharge which follows a single stimulus given to the normal portion of the muscle. If the veratrine concentration just produced a repetitive response (as in Fig. 7a) then the cathodal polarization increased the after-discharge following a driven action potential (Fig. 7b). In the experiment of Fig. 7 this increase was slight. As would be suspected, anodal polarization of the veratrinized stretch of muscle fibre subtracts from the effect of veratrine so that the addition of the anode to bath 2 stopped the after-discharge (Fig. 7d). The reverse effects were seen when the polarization of the bath containing veratrine was switched off. Immediately after removal of the cathode the veratrine after-discharge was decreased (Fig. 7c) ; after removing an anode the discharge in response to the single test stimulus increased (Fig. 7e). It is known that the polarizations forced on the membrane by passing current through it for ½ min. persist for a minute or so afterwards (Burns and Paton, 1951). Presumably the inward current flow due to the relatively slow collapse of super-polarization after withdrawal of an anode acts like the applied cathode, and adds to the inward current produced by the relatively slow repolarization of veratrinized membrane ; in this way the after-discharge is increased.

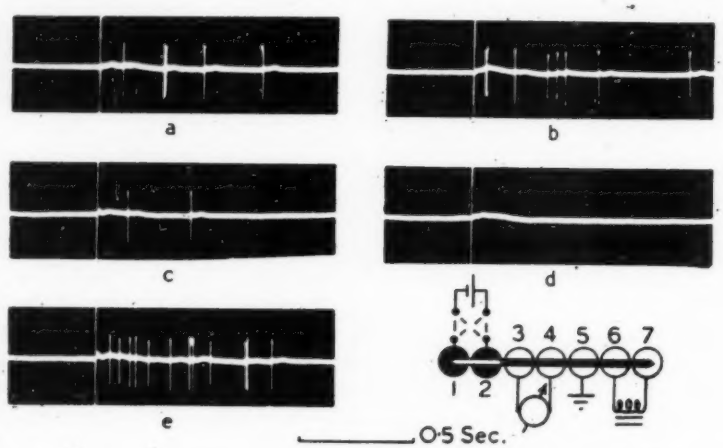

FIG. 7.—The responses to a single stimulus of (probably) one of a small bundle of muscle fibres during and after various polarizing currents in the presence of local veratrine. 2×10^{-7} veratrine sulphate in baths 1 and 2 only. Polarizing currents applied between baths 1 and 2. (*a*) Control. Polarizing current off. (*b*) 1 min. after switching on 7.5 μA current from bath 1 to bath 2. (Bath 1+). (*c*) 1 min. after switching current off. (*d*) 1 min. after switching on 7.5 μA current from bath 2 to bath 1. (Bath 2+). (*e*) 1 min. after switching off current.

DISCUSSION

In all of the electrically excitable tissues from which an after-discharge has been obtained veratrine appears to delay repolarization following an

action potential (Krayer and Acheson, 1946). It has been described by some as prolonging the negative after-potential; others have disliked this description on the grounds that an originally monophasic action potential can become one with two maxima of depolarization, repolarization thus occurring in two phases with a clear discontinuity between them. Whatever is the best way of describing events, repolarization is delayed by veratrine and in frog's muscle may occur with a time constant as great as 5 sec.

Although the mechanism by which veratrine delays repolarization is unknown, it is clear that the repetitive response to a single stimulus only occurs in the frog's sartorius muscle when the delay of repolarization is local. When veratrine is applied to the whole length of a muscle fibre, repolarization occurs equally slowly at all points along the fibre's length, and there is no cause for interstitial current flow. When the veratrine is applied locally, the veratrinized length of muscle fibre pursues the same slow time course of recovery as before, but current begins to flow in the surrounding fluid from the neighbouring and rapidly polarizing membrane toward the veratrinized region; it is this current flow which occurs during recovery from a driven action potential which may set up a "spontaneous action potential." The spread of this action potential, which is normally the first of a series, causes both veratrinized and normal membrane to depolarize a second time, following which the above cycle of events may be repeated, giving rise to the second action potential of the after-discharge. This is the characteristic mechanism of an after-discharge due to what has been termed differential repolarization (Burns, 1955).

Our results suggest that if the whole membrane of an electrically excitable cell is uniformly affected by veratrine, then no after-discharge can follow a driven action potential. On the other hand, after-discharges have been reported in nerve-muscle preparations submerged in veratrine solutions (Bacq and Brown, 1937) and can be obtained for many hours from nerve fibres which have been uniformly soaked in veratrine (Feng, 1941). It seems possible therefore that the mechanism of the drug's action is not the same for all susceptible electrically excitable cells. An alternative explanation of these findings may be that some excitable cells possess a membrane only certain parts of which are affected by the drug. Certainly some electrically excitable cells such as cardiac auricle do not show after-discharge in the presence of veratrine (Krayer and Acheson, 1946). In a nerve-muscle preparation, the region of the nerve terminals is known to be more sensitive than the proximal fibre (Dun and Feng, 1940). It was for this reason that we used tubocurarine-Ringer solution as a precaution against excitation through nerve twigs.

In our own experiments, the exposure of the whole length of the muscle fibres to veratrine solution lead to violent after-discharges following test stimuli, for the first 2 or 3 min. But these after-discharges always disappeared after a time interval consistent with the view that the concentration of the drug had finally become uniform at all points on the fibre's length. Consistent with this interpretation was the finding that a sudden decrease in veratrine concentration produced by the exchange of Ringer solution for veratrine solution would also cause after-discharges to test stimuli for a period of a few minutes.

The characteristics of an after-discharge due to differential repolarization have been described in connexion with the type-B cells of the cerebral cortex (Burns, 1954, 1955). Locally, veratrinized skeletal muscle seems to provide a simple biological model for the mechanism of this sort of after-discharge, and it could be said that the local application of veratrine converts the relatively phlegmatic muscle cell into a cell whose structure and function are as complex as those of some neurones found in the central nervous system. We have been able to demonstrate with veratrinized muscle many of the more important features of an after-discharge due to differential repolarization. Thus, when conditions are only just adequate for the production of repeated response to a single stimulus we found:

(*a*) An action potential starting from any point and travelling in any direction will trigger the after-discharge.

(*b*) The repetitive response tends to bear an all-or-nothing relationship to variation in the conditions required for its production. Because one "spontaneous" discharge resets the recovery cycle it produces the conditions necessary for a second "spontaneous" action potential; the second discharge produces a third, and so on. Thus the usual event is an after-discharge consisting of many action potentials and it is extremely hard to adjust conditions so that only one after-discharge occurs (Fig. 5).

(*c*) The latent period before the repetitive "spontaneous" firing breaks out is greater than the time interval between the first two action potentials of the after-discharge (Fig. 4).

(*d*) The maximum frequency of the repetitive response does not occur at the beginning of the after-discharge, but is usually reached after the first few action potentials (Fig. 4).

(*e*) When the after-discharge comes to an end its frequency does not decline asymptotically to zero, but after some reduction in the rate of discharge the series of action potentials stops suddenly (Fig. 4).

(*f*) The production of an after-discharge requires the presence of an adequate gradient of membrane potential along the length of the cell during recovery from driven activity (Figs. 2, 3, and 5).

These properties of after-discharge due to differential repolarization have been described at some length since they may ultimately serve to identify after-discharges of this type at other sites in the nervous system.

Locally veratrinized skeletal muscle offers a convenient preparation with which it should be possible to make a detailed analysis of the mechanism of this form of after-discharge. We have made no reference in this report to the precise site of origin of the after-discharge, other than to say that the origin is in the region of the border between veratrinized and normal muscle. The methods we have used in the experiments described are not adapted to precise localization, but the information available indicates that the after-discharge originates on the veratrine side of the vaseline junction separating veratrinized from normal Ringer solution. Because the vaseline junction is itself of the order of breadth of the muscle membrane's space constant, there must be gradients of both veratrine concentration and of recovery rate on either side of the vaseline barrier. This means that the point of slowest repolarization should be well inside the veratrinized bath. We are at present investigating this question in greater detail.

Summary

1. Experiments are described in which the effects of veratrine sulphate upon the fibres of the frog's sartorius muscle were tested.

2. Direct excitation of fully curarized muscle fibres gave rise to a repetitive after-discharge only when veratrine was not applied to the entire length of the fibres.

3. Veratrine delays repolarization of the muscle fibre's membrane after an action potential has passed through the veratrinized region.

4. The difference in repolarization rates of veratrinized and untreated lengths of muscle fibre is the immediate cause of the after-discharge.

5. The general properties of an after-discharge due to differential repolarization are discussed.

This work was supported by a grant from the National. Research Council of Canada, to whom we wish to express our thanks.

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INJECTIONS OF BULBOCAPNINE INTO THE CEREBRAL VENTRICLES OF CATS

BY

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(RECEIVED MAY 19, 1955)

Recently it was found (Feldberg and Sherwood, 1954b) that the anticholinesterases dyflos (DFP) and eserine, when given to cats by the intraventricular route, produce a condition with many of the features of "catatonia." Experimental "catatonia" or "catalepsy" in animals is usually associated with the drug bulbocapnine and was first obtained with this substance by Peters (1904). There are numerous later publications (for reference see Ingram and Ranson, 1934; de Jong, 1945) on the action of bulbocapnine in producing this condition when given subcutaneously, but relatively large doses, between 20 and 30 mg./kg., are required to bring about the effect when administered in this way. In the present experiments it is shown that bulbocapnine is active in much smaller doses by the intraventricular route.

METHODS

The experiments were performed on nine cats with a permanent cannula in the lateral cerebral ventricle. The method of implanting the cannula and of injection was the same as that described previously (Feldberg and Sherwood, 1953; 1954a). The bulbocapnine used was the hydrochloride, a sample of which was kindly given us by Dr. H. Molitor, of the Merck Institute, Rahway, New Jersey. The salt was injected in Tyrode solution. As the solubility in this neutral solvent is relatively poor at room temperatures, it was necessary to dissolve the salt in heated Tyrode solution and to keep the fluid warm until the injection was made. In this way it was possible to inject the doses used in a small volume (0.3 ml.) without having to acidify the solution. The only effect sometimes obtained by control injections of 0.3 ml. of saline or Tyrode solution was a tendency to sleepiness or subduedness developing 1 to 1½ hr. after the injection.

RESULTS

An effect was obtained regularly with 1 mg., and set in after a latency of 30 sec. to 3 min. During the latency some of the cats became agitated and manifested difficulty in locomotion; the gait became unsteady and there was occasional staggering and stumbling; some cats retched and vomited; one cat defaecated and had erection of the penis. After these preliminary signs all movements became severely restricted and slow, and the cats showed signs of "catatonia" in response to the usual tests. The onset of this change in behaviour was often so sudden that it overtook the cat during the execution of a movement which it did not complete. For instance, a cat would stop with one paw off the ground, half-way through a step. At this stage the cat usually assumed a crouching position with its legs flexed well under its body. This is apparently the position described by Ingram and Ranson (1934), in their experiments with subcutaneous injections of bulbocapnine, as "flexion position," "flexion tendency" and "flexion attitude," by which they refer to "the disposition of an animal to assume a crouched position with the limb flexed beneath it and the back somewhat arched." There is, at the beginning of the "catatonic" phase, a period in which the respiration rate is raised up to 40/min.

The "catatonic" phase was recognized by the following phenomena. When the cat was put on the ground, it remained standing in the position in which it landed for some time. When induced to walk, it stopped after a few steps, standing motionless for long periods before it gradually lay down in a crouched position. When the hind quarters were lifted from the ground and released after a few seconds, the cat took two or three steps on its front paws with its hind quarters still pointing upwards and the back concave before lowering its hind quarters. When a front paw was abducted or placed across the cat's own spine, it remained in this position for periods varying between 30 and 45 sec. When put into the erect posture with its front paws resting on the upper rungs of an inverted stool, the cat remained in this position for about 20 sec. before slowly climbing down. Similarly, a cat placed with its groin across the lower rung of an inverted stool remained in this

*With a personal grant from the Medical Research Council.

position for several seconds. The cat could be brought into a number of other unnatural attitudes by lifting its front or hind limbs, and it offered no resistance nor did it make any muscular effort to alter these positions. It had difficulty in extricating itself from awkward positions, but, when made to move or jump off a shelf, showed no signs of muscular incoordination.

The cat no longer reacted to events in the room, nor did it come forward when approached. A hand could be waved close to its face, eliciting nothing beyond a blinking of the eyes. It was also difficult to obtain the cat's attention by noises, but when stimulated by stroking, or inducing it to move, the cat betrayed its awareness by lashing its tail and changing its facial expression and, in several instances, by uttering hoarse cries which continued for some time after the stimulus ceased. Sometimes a cat uttered such hoarse cries of itself during the stage preceding "catatonia." The eyes were usually half shut, and an attempt to open the lids met with strong contraction of the orbicularis oculi muscles.

The "catatonic" phase was at its height 15–30 min. after the injection and gradually wore off during the following hour or two. This lightening of the animal's state was recognized by the fact that the cat could be made alert for short periods by persistent stimulation, that it was more easily induced to walk, or showed periods of spontaneous activity which were, however, still subject to sudden arrest and persistence in a pose.

With doses larger than 1 mg. there were additional features. In one cat, short-lasting general convulsions occurred during the first minute after an injection of 5 mg. bulbocapnine. Then followed a condition of deep "catatonia" lasting for over an hour. During this condition the cat, when hung on the upper rung of an inverted stool, supported itself with one forepaw only. Yet, when taken off the rung, it walked without signs of incoordination. Further, a hind leg twisted over its back returned only slowly to its normal position when the restraint was removed. When smoke was blown into the cat's face it made no withdrawal movements, but when touched it responded with howling or crying. When induced to step from one shelf to another, 3 in. higher, it ceased moving with one hind limb still remaining on the lower shelf, maintaining this position for minutes. At an early point in this "catatonic" state the cat, when undisturbed, lay flat on its belly and cried from time to time.

Another cat reacted to the intraventricular injection of 4 mg. bulbocapnine as follows. Within ½ min. it became unable to stand up. Although the legs were flexed, the claws were protruded and there was a pronounced grasp reflex, particularly on the forepaws. When a hind limb was touched there were contraction jerks of the limbs. In the course of about half an hour the cat regained the power to support itself; at first it walked clumsily, in an unsteady manner, and later on stiffly. When the usual tests for "catatonia" were applied it was found that the cat put up some perfunctory struggle before remaining in the abnormal position. Thus, when suspended with its forepaws on the upper rung of an inverted stool, it first struggled and began to move off but arrested this movement and remained fixed with one paw still holding on to the rung. It also struggled first when placed on both upper rungs of the inverted stool, but then remained there immobile until pushed off, when it jumped down and moved away without signs of incoordination. As with the other cat, under the effect of a large dose of bulbocapnine it showed some signs of awareness such as lashing its tail, changing its facial expression, and growling or purring according to the way it was handled.

Doses of bulbocapnine smaller than 1 mg. were too small to produce signs of "catatonia" consistently, but other striking changes in behaviour were observed.

One cat, for instance, during the first few minutes after an injection of 200 μg. of bulbocapnine had a laboured respiration; it vomited after some licking movements, salivated and retched. Ten to fifteen minutes later there was this pronounced change in the cat's previously docile and friendly behaviour: whenever approached it hit out with one or both front paws, sitting erect and hissing with bared teeth, without, however, following through with the attack. When left undisturbed, the cat paced about with quick and sudden movements, lashed its tail, and by its head movements in response to noises or tapping on the table betrayed heightened alertness. The animal was affected in this way for about 20 min. No signs of "catatonia" were produced by this dose of bulbocapnine. The same kinds of reactions —laboured respiration, vomiting, anger, but no feature of "catatonia"—were produced by the same dose on subsequent occasions.

In another cat the injection of 100 μg. immediately produced profound signs of "catatonia," as judged by the fact that the cat, or its limbs, could be placed without struggle in unnatural positions or attitudes which were retained for many seconds. In this state the cat often arrested the progress of

a movement and remained motionless in the posture thus acquired for minutes. When an attempt was made to dislodge a cat, on a broad shelf where it was being examined, by pushing against its shoulders or sides, it pressed in the opposite direction and anchored itself to the edge of the shelf with the paw of the appropriate side. The signs of "catatonia" were replaced after about 10 min. by behaviour characteristic of anger. Then, when an attempt was made to put its forepaw across its spine, the cat hit out and tried to bite, lashing its tail. This particular cat responded to a higher dose (200 μg.) with signs of intense itching and motor phenomena of convulsive quality. The cat started to scratch itself, to wipe and lick its paws with increasing vigour, until this activity became paroxysmal. Movements were ill controlled and jerky. The gait was unsteady and spastic, there was often typical scissor gait; the claws tended to close over any object and it was difficult to disengage them. The legs were extended and the basic posture was a crouching one with the body close to the ground; from time to time the cat rushed forward or backward, or circled in either direction, suddenly coming to a halt with eyelids and ears twitching. Within 20 min. the cat gained control over its movements and was then easily startled.

Discussion

Bulbocapnine, when injected intraventricularly, produces its characteristic effects in doses a fraction of those effective on subcutaneous injection. With both methods of application the effects are very similar, except that with intraventricular injection the effect sets in earlier and disappears more quickly than with subcutaneous injection. The time taken for absorption from the subcutaneous tissue explains this difference in the onset and duration of the effects.

With the present method of administration the relative smallness of the dosage and the speed with which the bulbocapnine effects appear indicate that structures close to or directly in contact with the ventricular surface are likely to be implicated. This indication is borne out by comparison with results obtained by lesions and stimulation of selected anatomical sites in the mid-brain close to the ventricular lining. From these, and only from these sites, effects analogous to those of bulbocapnine are obtained.

There are many observations (Ingram, Barris, and Ranson, 1936; Bailey, 1948; Magoun, 1952; MacCulloch, Ridley, and Sherwood, 1952; Hess, 1954) that lesions on the border between the upper tegmentum and the posterior hypothalamus produce a condition resembling the "catatonic stupor" of bulbocapnine. Moreover, not only "catatonic stupor" but also other signs such as those of anger and irritability, described here after bulbocapnine, have been elicited from these regions.

In his classical experiments with electrical stimulation of the diencephalon, Hess obtained hissing, snarling, excitement, and calling, apparently of a plaintive quality, with stimulation through electrodes in the following areas: the wall of the third ventricle from the level of the subthalamus above to the infundibulum below, including points close to the descending column of the fornix and the tracts of Vic d'Azur and an area extending caudad in the periaqueductal grey and the medial longitudinal bundle. Further, when lesions had been made by electro-coagulation in the area immediately above the mammillary bodies and rostral to the red nuclei, Hess obtained not only the typical signs of catatonic stupor, but some cats became hostile as the catatonic stupor wore off during subsequent weeks. A previously tame cat with a lesion in the posterior hypothalamus, bordering the tegmentum and involving the lining of the third ventricle as far caudad as the entry of the aqueduct, would hiss and hit out when touched or approached and then would move away in a crouching position without following up the attack. Another cat with a lesion only slightly further caudad and lateral to that in the previous cat, but this time not destroying the ependyma, uttered continuous plaintive cries when observed until it died three weeks later. Similar behaviour was seen by Sherwood, Ridley, and McCulloch (unpublished) in cats with lesions in the upper brain stem. The changes of behaviour in these cats recalls in a striking fashion those seen after intraventricular injections of bulbocapnine.

On intraventricular injection the anticholinesterases eserine and DFP regularly, before the onset of catatonic stupor, produced intense scratching, washing, and licking of the paws, giving the impression of itching (Feldberg and Sherwood, 1954b), an effect also obtained in one experiment with bulbocapnine. Hess found that cats acted thus on stimulation of the anterior intraventricular portion of the fornix, of the nuclei of the septum pellucidum as far ventrally as the anterior commissure, and of a region immediately above and behind the mammillary bodies—again structures bathed by the ventricular fluid. From some areas the effect was obtained during stimulation, from others immediately after it.

In animals, the main method of obtaining the syndrome of catatonia, apart from drugs, is the

placing of lesions in the upper brain stem. This raises the question whether bulbocapnine also produces a kind of pharmacological lesion by paralysing nerve cells and thereby interrupting some specific pathways, in analogy to the paralysing effects of nicotine or acetylcholine on peripheral structures when producing block of synaptic transmission in a sympathetic ganglion, or neuromuscular block. In this connexion it may be recalled that large doses of acetylcholine as well as of eserine and DFP will produce catatonia when injected intraventricularly.

The finding that in relatively large doses bulbocapnine, on intraventricular injection, evokes convulsions, as it does on subcutaneous injection, renders it likely that the convulsions are also of subcortical origin. The site of action may well be the thalamus, stimulation of which with high voltages is known to produce seizures of all kinds (Jasper and Droogleever-Fortuyn, 1947; Hunter and Jasper, 1949; Hunter, 1950; Kaelin, 1951). In its convulsive action on intraventricular administration, bulbocapnine has an effect in common with (+)-tubocurarine, acetylcholine, DFP, and eserine.

Summary

1. Intraventricular injections of 1 mg. bulbocapnine produce in cats a state of catatonic stupor; to produce this condition with subcutaneous injections doses at least forty times larger are required.

2. Intraventricular injections of 100–200 μg. of bulbocapnine which need not evoke catatonic stupor produce short-lasting changes in behaviour: a previously docile cat may show hostility.

3. The changes produced by bulbocapnine on intraventricular injections are correlated with similar changes obtained from electrical stimulation and from lesions at discrete points in the mid-brain.

The detailed description of the experiments of Professor W. R. Hess given in the Discussion was obtained during a visit by the authors for the purpose of working in his extensive "Brain Biological Collection" which is at present accommodated in the Anatomical Institute of the University of Zurich and consists of films, protocols, and histological preparations from his experiments. We should like to record our great indebtedness to Professor Hess for the hospitality and generous help we received.

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Brit. J. Pharmacol. (1955), **10**, 375.

THE PHARMACOLOGY OF AN IRON-DEXTRAN INTRAMUSCULAR HAEMATINIC

BY

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(RECEIVED MAY 19, 1955)

An iron preparation suitable for intramuscular injection should conform to the following criteria: (*a*) it should be rapidly absorbed from the muscle and the total iron content should be available for haemoglobin synthesis, (*b*) in order to avoid pain, the *p*H and tonicity should approximate to those of normal tissue fluids, (*c*) it should be stable in the presence of protein and electrolytes, (*d*) it should possess a low toxicity, and be stable on storage, (*e*) in order to keep the injection volume down to a minimum, the solution should contain at least 5% w/v of iron.

Previous workers have described the subcutaneous or intramuscular administration of compounds containing either ionic iron (Brownlee, 1942; Goldberg and Hutchison, 1953) colloidal ferric hydroxide, or saccharated oxide of iron (Slack, 1949), but preparations which contained sufficient iron to be useful in the treatment of hypochromic anaemia were found to be either toxic or painful.

Initially, we investigated two series of iron compounds. In the first series, the iron was present as a co-ordination compound, e.g., ferrous calcium ethylenediaminetetraacetate, and other ferrous or ferric alkali, alkaline earth, or organic base salts of ethylenediaminetetraacetic acid. In the second series, the preparations were based on saccharated oxide of iron, but other sugars were used in place of sucrose. On investigation in animals, compounds of the first series caused systemic haemorrhage and were rejected; none of the preparations in the second series showed satisfactory absorption from muscle, and some were, in addition, very toxic.

Iron is stored in the body as a macromolecular protein complex, ferritin, and is also transported in combination with the protein, β-globulin. It was decided, therefore, to investigate the substitution of the protein moiety by other macromolecules; a third series was therefore investigated in which the iron was combined with polysaccharides or their partial degradation products. Encouraging results were only obtained with a low molecular dextran, and, using this, an intramuscular iron preparation has been made which satisfies the foregoing criteria, in that it is well absorbed, has a low toxicity, and is a satisfactory haematinic (Fletcher and London, 1954). An account is presented of the pharmacology of this low molecular dextran-iron complex, using saccharated oxide of iron as a basis of comparison.

METHODS

1. A solution of low molecular dextran-iron complex containing 5% w/v of iron (Imferon),* with a *p*H of 6.

2. Saccharated oxide of iron. Two different preparations, each containing 2% w/v of iron, designated A (*p*H 10.76) and B (*p*H 9.6) were investigated.

In vitro *Precipitation of Ferric Hydroxide.*—This was based essentially on the work of Nissim and Robson (1949); in addition, the stability was investigated at an iron concentration (0.004% Fe) in the therapeutic range.

Paper electrophoresis was carried out on strips of 3 MM paper in an acetate buffer at *p*H 5 and a barbitone buffer at *p*H 8.6. The potential applied ranged from 100–1,000 volts, and the time from 6–24 hr. Low molecular dextran was used as an indicator of electro-osmosis.

Anticoagulant Activity.—This was measured *in vitro* by the method of Nissim (1954), but the blood was added directly to the tubes from a cannula inserted into the carotid artery. The clotting time *in vivo* was determined by the Lee and White method on samples of blood obtained by heart puncture.

Haemolytic Effect.—The method of Nissim (1954) was used, but the degree of haemolysis was determined by counting the remaining red cells.

* Patent applied for in United Kingdom and oversea territories.

Toxicity.—Albino mice were maintained on MRC diet 41 and allowed free access to water. The acute toxicity of each compound was determined after intravenous administration to groups of mice. The number of survivors in each group was counted on the tenth day after injection and the LD50 calculated by plotting the percentage mortality against the dose on logarithmic probability paper. The small size of the leg muscles of the mouse made it impracticable to administer intramuscularly more than 9.5 ml./kg. of the preparations. Doses of iron-dextran of up to 650 mg. Fe/kg. were injected intramuscularly into both hind legs of rabbits and the animals observed for six months after the injection. Chronic toxicity was studied in rats fed MRC diet 41 and rabbits fed MRC diet 18, which were given daily doses of iron-dextran over long periods.

Antigenicity.—Five guinea-pigs of 400–500 g. bodyweight were "sensitized" with 1.0 ml. of iron-dextran/kg. bodyweight intravenously and given a challenging dose of 1 ml./kg. bodyweight 14 days later. Three preparations of iron-dextran made by the same method were investigated in rabbits. Two rabbits were injected 18 times with 0.5 ml. of each preparation during a period of two months. A week later, samples of blood were taken and the sera subjected to precipitin and complement fixation tests with dextran polymer solutions over the range 10^{-2} to 10^{-4} dilution and with iron-dextran solutions over the same range of dilution.

Diffusion of iron after intravenous injection was investigated by determining the change in serum iron concentration in rabbits and the increase in the iron concentration in the peritoneal fluid in mice. Samples of blood were obtained from the marginal ear vein in rabbits. The mice were injected slowly with 200 mg. Fe/kg. of each compound and the concentration of iron in the peritoneal fluid determined (Nissim, 1953a).

Excretion of Iron in Urine and Faeces.—Rabbits were catheterized, injected intravenously with the preparation, placed in metabolism cages, and the urine collected during 24 hours, after which the animals were again catheterized and the samples added to the previously voided specimens. The faeces were collected during two 24-hour periods before injection, and at the same intervals up to four days after injection.

Determination of Intramuscular Absorption of Iron from Site of Injection.—The iron preparations were injected deep into the glutei of rabbits. The animals were sacrificed at definite time intervals after injection. The injected leg was amputated and the residual iron determined. The contralateral leg was used as a control.

Estimation of Iron.—Serum: The method of Laurell (1947) was used, but the HCl concentration was increased to 6 N in order to ensure complete breakdown of the complex prior to colorimetric estimation with either $\alpha\alpha'$dipyridyl or potassium thiocyanate (Ventura and Klopper, 1951). *Muscle, urine, and faeces:* These were wet-ashed with nitric and sulphuric acid and the iron determined colorimetrically by means of potassium thiocyanate.

Haemoglobin was estimated by the method of King (1951).

Iron utilization was studied in 140 suckling piglets. The decline in haemoglobin was followed during the first week to 10 days of life; when the haemoglobin had fallen to approximately 50%, the iron-dextran was administered in a dose of 26 mg. Fe/kg. The haemoglobin was then determined at weekly intervals.

Results

In vitro *Precipitation of Protein.—Serum:* 0.05 ml. of each iron preparation was added to 1 ml. of serum and the mixture incubated for 2 hr. at 37° C. No precipitation of protein was observed with either the saccharated oxide of iron preparations or the iron-dextran. *Fibrinogen solution:* The following serial dilutions were prepared: iron-dextran, 5.0–0.125% Fe; saccharated oxide of iron, 2–0.125% Fe. To 0.3 ml. of each test solution was added 1 ml. of a 1% bovine fibrinogen solution, and the solution was kept for 2 hr. at room temperature. None of the preparations precipitated fibrinogen.

Precipitation of Ferric Hydroxide.—The *p*H at which ferric hydroxide is precipitated from a saccharated oxide of iron solution varies with the type of preparation (Table I). The *p*H of iron-dextran was reduced from 6 to 1, but no ferric hydroxide was precipitated.

TABLE I

*p*H OF PRECIPITATION OF FERRIC HYDROXIDE

Medium	Final % Fe	Iron-dextran	Saccharated Oxide of Iron A'	Saccharated Oxide of Iron B
Water ..	0·004	No precipitation down to *p*H 1	5·9	3·7
,, ..	0·1	,, ,,	6·0	3·7
Rabbit plasma	0·1	,, ,,	7·7	5·8

Paper Electrophoresis.—Saccharated oxide of iron moved towards the anode, and is, therefore, a negatively charged molecule. The iron-dextran moved in the same direction as the dextran marker, but at a slightly slower rate, and it would appear that the iron-dextran molecule is feebly negatively charged. Dr. N. J. Berridge, of the National Institute for Research in Dairying, Reading, has determined the charge of iron-dextran in a Tiselius apparatus and also found it to be negatively charged in a 0.1M phosphate buffer at *p*H 6.8.

Tonicity was determined in a modified Beckmann apparatus. The iron-dextran was dissolved in normal saline and the solution had a freezing point depression of 0.9° C.

Anticoagulant Activity.—Iron-dextran is considerably less anticoagulant *in vitro* than saccharated oxide of iron (Table II). In rabbits after intravenous doses of up to 50 mg. Fe/kg., i.e., 10 times the therapeutic dose, the clotting time was not significantly elevated.

TABLE II

IN VITRO ANTICOAGULANT ACTIVITY OF IRON-DEXTRAN

1 ml. of blood added to 0.32 ml. of solution of iron

Concentration of Fe %	Clotting Time (min.)		
	Saccharated Oxide of Iron	1% NaOH	Iron-dextran
1·0	45*	95*	20
0·5	40*	>240	9
0·25	28	27	9
0·125	20	13	9
0·0625	16	11	8
0·0312	11	11	7
0·0160	8	11	7
0·0080	7	11	5
Control	5	11	5

* It was not possible to obtain an accurate end-point because of haemolysis and partial clotting.

Haemolysis

The haemolysing effect of the two iron preparations was studied *in vitro*. Iron-dextran showed a gradual decline in haemolytic activity from 2 to 0.5% Fe. Below 0.25% Fe no haemolytic activity was detected. Saccharated oxide of iron caused complete haemolysis down to 0.25% Fe but no haemolysis below 0.125% Fe.

Acute Toxicity

The LD50 after intravenous injection into mice was $1,013 \pm 81$ mg. Fe/kg. for iron-dextran. Both preparations of saccharated oxide of iron had an LD50 of 231 ± 64 mg. Fe/kg. Both iron-dextran and saccharated oxide of iron proved more toxic after intravenous administration to rabbits than to mice. Two rabbits which were injected with iron-dextran at a dose equivalent to 500 mg. Fe/kg. died 9 days after injection. Four rabbits injected intravenously with the saccharated oxide of iron solution, preparations A and B, equivalent to 150 mg. Fe/kg., died between 6 and 24 hr. after injection. It has not been possible to reach a lethal dose-level when iron-dextran is injected intramuscularly into mice (maximum practicable dose 450 mg. Fe/kg. bodyweight); rabbits survived at 690 mg. Fe/kg. bodyweight intramuscularly.

Chronic Toxicity

Rats.—A group of 8 rats which received a total dose of iron-dextran equivalent to 100 mg. Fe/kg. bodyweight in divided doses of 2 mg. Fe/kg. over a 12-week period showed a weight gain comparable with that of normal controls and at autopsy all the organs appeared normal. The tissue/bodyweight ratios for liver, spleen, and kidneys were 11, 3, and 5% higher than those of untreated control animals. A further group of 8 rats on a higher dosage equivalent to 1,000 mg. Fe/kg., administered over a similar period, showed at autopsy enlarged livers and spleens, the tissue/bodyweight ratios in this instance being increased by 87% for liver, 50% for spleen, but only 2% for kidneys.

Rabbits.—A total dose of 1,060 mg. Fe/kg. bodyweight was given either intramuscularly or intravenously to groups of 4 rabbits in divided doses of 20 mg. Fe/kg. over a period of 3 months. Satisfactory weight gains were maintained over the first 9 weeks, but a slight falling-off compared with normal controls occurred towards the end of the period.

The rabbits were sacrificed and autopsy on those which had received the intravenous injections revealed enlarged liver, spleen, and kidneys, brown stained mesentery and excess pleural fluid, the tissue/bodyweight ratios being liver 96%, spleen 48%, and kidneys 21%, respectively, in excess of normal controls. In the rabbits which had been given iron intramuscularly, hepatomegaly was less prominent, while spleen and kidneys were reduced in weight (tissue/bodyweight ratios $+41\%$, -33%, and -11% respectively).

A full account of the histological examination of the tissues from these animals will be published elsewhere. Suffice it to say here that the general appearance of organs such as the liver, spleen, and adrenals had much in common with the descriptions published by Cappell (1930) and Nissim (1953b). The lungs constituted a striking exception, since not only were they free from haemorrhages but iron deposition was slight, even after the heaviest and most prolonged dosage. No doubt this is a reflection of the stability of iron-dextran and is one of the reasons for its low toxicity. Such iron as was present in the lungs was confined to the connective tissue surrounding arteries and the cartilage plates of bronchi.

The appearance of the muscles at the sites of injection is of interest. With iron preparations which are poorly absorbed, such as saccharated oxide of iron, extensive brown discoloration is observed. With iron-dextran the brown staining

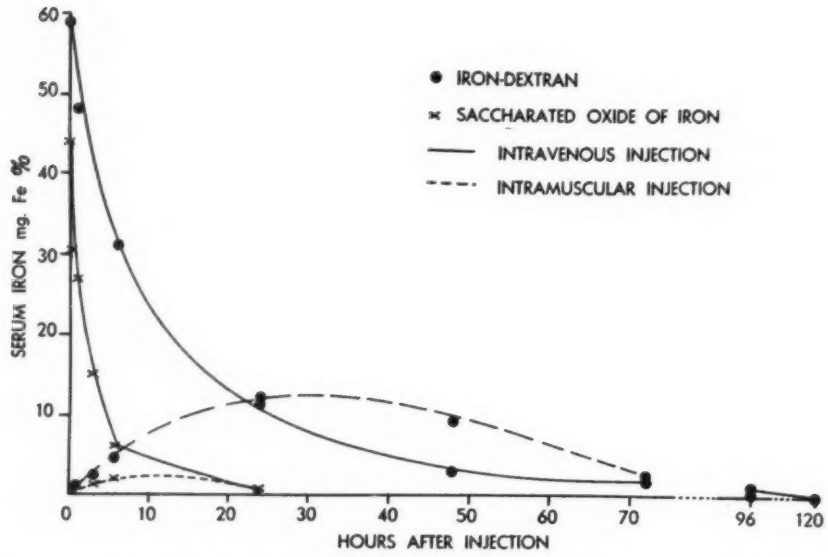

FIG. 1.—Serum iron levels in rabbits after different iron preparations administered i.v. and i.m. in a dose equivalent to 20 mg. Fe/kg.

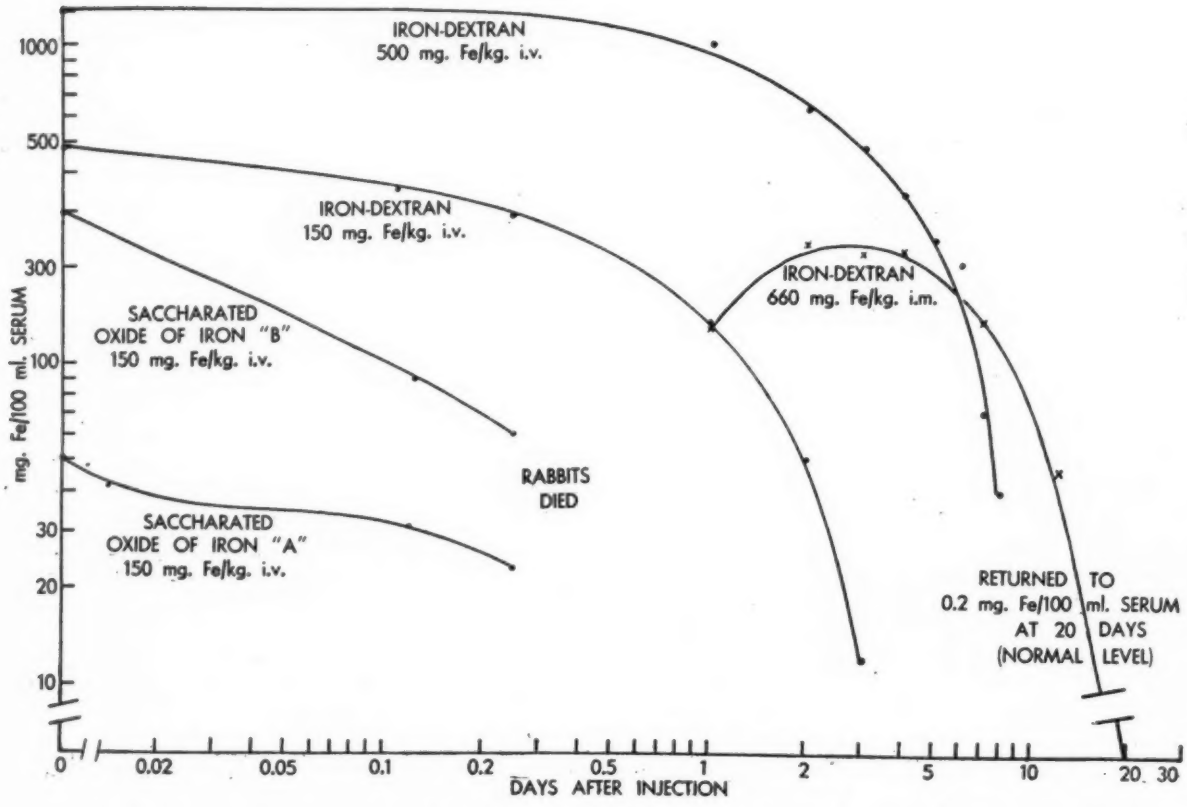

FIG. 2.—Serum iron levels in rabbits after different iron preparations administered i.v. and i.m. at varying dose levels.

was minimal and often wholly absent. In section the muscle fibres appeared normal, but considerable iron deposition could be demonstrated in the connective tissue between the fibres and in the endothelial cells of some capillaries.

Antigenicity

There were no signs of anaphylaxis in guinea-pigs when the challenging dose was administered 14 days after sensitization. In no rabbit, after injecting each 18 times with 0.5 ml. of iron-dextran during two months, has it been possible to demonstrate any antigenic properties for the preparation in that no serum antibodies were produced which would react with either dextran or iron-dextran.

Diffusion of Iron after Intravenous Injection

From Fig. 1 it can be seen that iron-dextran is much more slowly removed from the blood stream than an equivalent dose of saccharated oxide of iron. When the dose level was increased to 150 mg. Fe/kg. (Fig. 2), the serum iron concentration in the 5 min. sample of saccharated oxide of iron preparation A was only 51 mg.% (average 2 rabbits), with preparation B it was 308 mg.%, whereas after iron-dextran it was 431 mg.% (average 2 rabbits). The rabbits on both saccharated oxide of iron preparations died shortly after the 6 hr. sample was collected. Serum iron concentrations were determined in rabbits which received a dose intravenously of iron-dextran equivalent to 500 mg. Fe/kg. bodyweight (Fig. 2). All three animals which received this dose died between the 8th and 9th day after injection. Saccharated oxide of iron and iron-dextran were

TABLE III

DIFFUSION OF IRON INTO THE PERITONEAL CAVITY OF MICE AFTER I.V. INJECTION

Preparation Injected	Dose in Terms of Iron	Fe Concentration mg./100 ml. in Peritoneal Fluid 10 min. after Injection
Iron dextran ..	200 mg./kg.	0·556 mg.
Saccharated oxide of iron	200 „	0·374
Saline control ..	Nil	0·227

administered to mice by slow intravenous injection in doses of 200 mg. Fe/kg. In each case the iron concentration of the peritoneal fluid was estimated, and similar values were obtained (Table III).

Excretion of Iron into the Urine and Faeces

The urinary excretion of iron after the administration of varying doses of iron-dextran is comparable with that of saccharated oxide of iron (Table IV). The mean faecal iron of the 2 rabbits

TABLE IV

EXCRETION OF IRON INTO THE URINE AFTER INTRAVENOUS INJECTION

Compound	Dose (mg. Fe/kg.)	mg. Fe Excreted in 24 hr.	% of Dose Excreted
Iron-dextran ..	20	0·48	0·5
	50	0·5	0·5
	500	1·52	0·12
Saccharated oxide of iron A ..	50	1·3	1·3

Control 24 hr. urine. 0.032 mg. Fe/24 hr.

which received iron-dextran at a dose equivalent to 500 mg. Fe/kg. was 21 mg. Fe/24 hr. before injection and 17.5 mg. during the 24 hr. period after the injection. During the 24–48 hr. period the animals developed diarrhoea and the faecal iron rose to 40.1 mg., at 48–72 hr. it was 36.9 mg., i.e., 2.6% of the total dose of iron was excreted during the first 72 hr. after injection. The diarrhoea increased in severity until death occurred and it was impossible to collect satisfactory faecal samples after the third day. Animals which received 20 mg. Fe/kg. of iron-dextran showed no increase in faecal iron during the four days after injection.

Absorption of Injected Iron from Site of Administration

The percentage absorption of each of the compounds studied when administered intramuscularly at a dose of iron equivalent to 20 mg. Fe/kg. is given in Table V. In rabbits which received iron-dextran at a dose equivalent to 690 mg. Fe/kg.,

TABLE V

ABSORPTION OF IRON AFTER INTRAMUSCULAR INJECTION

Dose: 20 mg. Fe/kg.

Compound	Time after Injection (Days)	
	2	56
Iron-dextran	87·5% (6)	98% (4)
Saccharated oxide of iron ..	10% (2)	47% (7)

No. of rabbits shown in parentheses.

the residual iron in the muscle 42 days after the injection was 2%—i.e., 98% of the dose of iron administered had been absorbed. The absorption of iron-dextran from the muscle can be followed by the resulting increase in serum iron concentration.

Elevation of Serum Iron after Intramuscular Injection

The increments in serum iron obtained after doses of each of the preparations equivalent to

20 mg. Fe/kg. bodyweight are compared in Fig. 1. Saccharated oxide of iron, which is only poorly absorbed, produces a slight initial elevation of the serum iron which is not maintained, whereas the serum iron concentration a few hours after administration of iron-dextran is elevated well above that of the iron binding capacity of the plasma protein, and the serum colour becomes progressively darker, making it likely that the iron is being absorbed as iron-dextran. The serum iron concentration continues to increase, reaching a maximum during the 24–48 hr. period. In rabbits which received intramuscularly a dose of iron-dextran equivalent to 660 mg. Fe/kg. the serum iron concentration reached a maximum on the second day after injection, when it was of the same order as that obtained immediately after an intravenous dose of 100 mg. Fe/kg. of iron-dextran. The level remained constant until the fifth day ; presumably during this period the rate of absorption of iron into the plasma equalled the rate of removal by the tissues.

Iron Utilization

Litters of domestic piglets, which suffer from a naturally occurring hypochromic anaemia during the first weeks of life, showed a remarkably rapid response to intramuscular injections of iron-dextran. A typical litter of 8 piglets had an average haemoglobin level of 5.1 g.% with a total circulating content of 21.6 g. before treatment. Following an average dose of 140 mg. of iron-dextran, the haemoglobin level rose to 9.6 g.% (48.3 g. circulating) within 7 days and 9.8 g.% (60.8 g. circulating) at 14 days—that is, 63.5% and 93.5% utilization respectively (McDonald, 1955).

Discussion

Iron-dextran has one-third of the acute toxicity of the best type of saccharated oxide of iron preparation ; the latter was the least toxic iron preparation previously described. In animals, iron-dextran has been shown to be innocuous when given intravenously or intramuscularly, but in man its prime use is as an intramuscular preparation. The mechanisms by which iron exerts its toxic effect are complex, but the difference in toxicity between the two types of preparation can be correlated with their different stability, anticoagulant activity, and haemolytic effect. The stability of a colloid is a function of its charge (ζ potential) and degree of solvation.

Electrophoretic measurements indicate that iron-dextran is a feebly negatively-charged molecule which does not depend for its stability on an adsorbed layer of strongly charged ions, as does saccharated oxide of iron. In this manner the stability of iron-dextran in the presence of ions would be accounted for. Thus the low molecular dextran appears to act as a protective lyophilic colloid. Saccharated oxide of iron contains 1% of NaOH, and the colloid is stabilized by hydroxyl ions so that neutralization of the negative charge causes precipitation of ferric hydroxide. The *p*H at which precipitation takes place varies with the type of preparation ; of the two which were tested, the more stable preparation B precipitated from aqueous solution at *p*H 3.7, and from plasma at *p*H 5.8. The difference in stability of the two preparations observed *in vitro* is also found *in vivo*.

When saccharated oxide of iron *p*H 9.6–11 is administered intravenously the resulting reduction in *p*H lowers the stability of the colloid ; this may be the reason for the pulmonary haemorrhage, and precipitation of iron, described by Cappell (1930) and by Nissim (1935b) after intravenous doses of saccharated oxide of iron greater than 45 mg. Fe/kg. Iron-dextran does not precipitate with change of *p*H, and therefore the buffering action of the blood does not appear to affect its stability.

Saccharated oxide of iron preparations administered intravenously are rapidly removed from the circulation (Figs. 1, 2). There is a negligible increment in peritoneal fluid iron content, and only 1–2% of the iron is excreted into the urine ; the iron is largely removed by direct uptake by cells of the reticulo-endothelial system. When the dose of saccharated oxide of iron is increased to 150 mg. Fe/kg., the iron concentration immediately after injection is lower than that calculated from the plasma volume of the animal (Nissim, 1953). All the rabbits which received this dose of iron died ; pulmonary and systemic haemorrhages were noted at autopsy.

Iron-dextran is more slowly removed from the plasma than saccharated oxide of iron, and animals which received 150 mg. Fe/kg. survived. The immediate serum iron concentration after doses of up to 500 mg. Fe/kg. was of the order expected from the plasma volume of the rabbit. The rate of diffusion of the iron into the peritoneal fluid, and the urinary and faecal iron excretion, are of the same order as those found after administration of saccharated oxide of iron.

The *in vitro* anticoagulant activity of iron-dextran, as determined by the method of Nissim (1954), is considerably less than that of saccharated oxide of iron. The sodium hydroxide present in

the latter shows marked *in vitro* anticoagulant activity. Hence, it is difficult to assess what contribution is made by the iron sucrose combination. The alkali is rapidly neutralized when the preparation is administered to animals, and it would not therefore contribute to *in vivo* anticoagulability. Ten times the therapeutic dose of iron-dextran given intravenously to rabbits has not elevated the clotting time.

The formation of antigens by absorbing polysaccharides on colloids has been reported (Zosaya, 1932). With iron-dextran no evidence of antibody production was found in rabbits, nor was it possible to produce anaphylactic shock in guinea-pigs with the preparation.

Brownlee (1942) investigated the absorption, after subcutaneous injection to rats, of a series of iron compounds and reported that iron triethanolamine chelidamate was a satisfactory haematinic. Slack (1949) administered this compound intramuscularly in doses of the equivalent of 7–14 mg. Fe per patient. The patients experienced severe pain and treatment had to be discontinued. Reports (Thomas, 1954 ; Munro, 1954) have been published that saccharated oxide of iron can be administered intramuscularly, but studies in rabbits have revealed that it is only partially absorbed. This may be due to neutralization of the alkali by the tissue fluid, with the resultant precipitation of ferric hydroxide which is not absorbed.

Iron-dextran is stable in the presence of tissue fluids, and is well absorbed after intramuscular injection. The complex is apparently absorbed unchanged, and intramuscular injection of this compound is perhaps comparable with a slow intravenous injection. Up to the equivalent of 1.3 g. Fe has been absorbed in rabbits after intramuscular injection. The complex is only slowly removed from the circulation, and is taken up by the reticulo-endothelial system, where the iron is made available for haemoglobin synthesis. Piglets suffering from iron deficiency anaemia utilized, during the first 14 days after intramuscular injection, 93% of the dose of iron administered. In cases of iron deficiency anaemia in man, the utilization of the iron after intramuscular injection has been of the same order as that described after the intravenous administration of saccharated oxide of iron (Fletcher and Fee, 1953 ; Baird and Podmore, 1954 ; Scott and Govan, 1954 ; Cappell, Hutchison, Hendry, and Conway, 1954 ; Jennison and Ellis, 1954).

Summary

1. A study has been made of the pharmacology of an iron-dextran complex (" Imferon ") and a comparison drawn with saccharated oxide of iron.

2. Iron-dextran is more slowly removed from the blood than is saccharated oxide of iron. The serum iron immediately after a dose of iron-dextran equivalent to 500 mg. Fe/kg. corresponds to the theoretical value based on even distribution in the plasma. With doses of saccharated oxide of iron equivalent to 150 mg. Fe/kg. the serum iron concentration immediately after injection was lower than the theoretical value.

3. Section of lungs from animals which received doses of saccharated oxide of iron equivalent to 150 mg. Fe/kg. revealed heavy iron precipitation and haemorrhages. Only slight deposition of iron was observed in the lungs from animals which received up to 500 mg. Fe/kg. of iron-dextran, and the lungs were free from haemorrhage.

4. The acute toxicity in mice of iron-dextran is only of the order of one-third that of saccharated oxide of iron.

5. The urinary and faecal iron excretions are of the same order as those described for saccharated oxide of iron.

6. After intramuscular injection, iron-dextran is rapidly absorbed, the iron being taken up by the reticulo-endothelial system. Muscle absorption studies on saccharated oxide of iron, up to two months after injection, revealed that it was only partially absorbed, and pronounced brown staining of the muscle persisted.

7. Anaemic piglets utilized, during the first 14 days after intramuscular injection, 93% of the dose of iron administered.

The authors wish to thank Dr. L. Golberg, director of research, for undertaking the histology and for assistance in the preparation of this paper ; Mr. C. Epstein and Miss P. Jewell for carrying out the antigenicity experiments ; Miss J. Leeson and Miss E. Beard for technical assistance ; and Dr. N. J. Berridge for determining the charge of iron-dextran by the Tiselius electrophoresis method. Thanks are also due to the board of directors of Benger Laboratories Ltd. for permission to publish.

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THE PHARMACOLOGICAL PROPERTIES OF THE OPTICAL ISOMERS OF BENZHEXOL, PROCYCLIDINE, TRICYCLAMOL AND RELATED COMPOUNDS

BY

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(RECEIVED MAY 25, 1955)

The powerful peripheral atropine-like ("antimuscarinic") actions of benzhexol, procyclidine, and its quaternary derivative tricyclamol, are described by Cunningham, Harned, Clark, Cosgrove, Daugherty, Hine, Vessey, and Yuda (1949), Montuschi, Phillips, Prescott, and Green (1952) and Lee, Gibson, Dinwiddie, and Mills (1954) respectively. The atropine-like properties of related quaternary amino*cyclo*hexyl-carbinols have also been mentioned (see Adamson, Barrett, and Wilkinson, 1951). Benzhexol and procyclidine are used for symptomatic relief in Parkinson's disease and tricyclamol is used for its spasmolytic action on smooth muscle.

Only the racemic forms of the above compounds have so far been described. We have separated the optical isomers and have shown that the antimuscarinic action is largely confined to the *laevo* isomers, most of which are not more toxic than the corresponding *dextro* isomers, and do not differ greatly from them in certain other respects.

METHODS

Chemical

Resolution was effected by crystallization of the acid D tartrates from ethyl alcohol, successive crops of solid being removed until only oily material would separate. Ether was added as necessary to facilitate crystallization. The solid consisted chiefly of *dextro* base D tartrate with the *laevo* base D tartrate remaining in the oil and mother liquor. Each was made alkaline with ammonia and the base crystallized from ethanol. The first crops were always of pure optically active base, later crops being of the racemic form.

The hydrochlorides crystallized from ethanol-ethyl acetate mixture.

The quaternary salts were obtained by addition of methyl or ethyl iodide to a solution of the appropriate base in acetone. They were recrystallized from acetone.

Pharmacological

The two optical isomers and the racemic mixtures of each compound were simultaneously compared with atropine by the mydriatic test in mice (Ing, Dawes, and Wajda, 1945), and by their activities in relaxing ACh-induced spasm of isolated guinea-pig ileum. In the mydriatic tests activity was estimated from the pupil diameters 30 min. after intraperitoneal injection of two or three doses of each compound, using groups of ten mice. The ileum tests were carried out in the manner previously described (Green, 1953) but using ACh (5×10^{-7}) to induce spasm. LD50's were determined by intravenous injection in groups of white Swiss mice, using a 2:3 dose ratio.

Vagal bradycardia or contraction of the nictitating membrane was elicited in cats under chloralose anaesthesia by submaximal stimulation of the cut vagus or cervical sympathetic nerves drawn into fluid electrodes. The stimulus consisted of D.C. pulses at 20 cycles/sec. from a blocking oscillator (Dickinson, 1950), applied for periods of 3 or 5 sec.

The peristaltic reflex of isolated guinea-pig ileum was recorded by Trendelenburg's method and elicited by raising the internal pressure to 2.5 cm. water.

Pain thresholds in the analgesic tests were determined by the pressure method of Green and Young (1951).

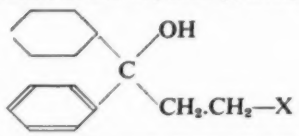
RESULTS

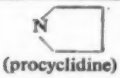
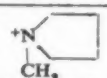
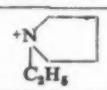
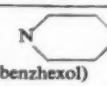
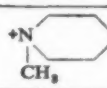
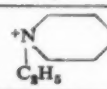
The activities of the *laevo* isomers, tested by mydriasis in mice or relaxation of ACh-induced spasm of guinea-pig ileum, were about twice those of the *racemic* mixtures, and far greater than those of the respective *dextro* isomers, except for the ethiodide of procyclidine, where the differences in activity were relatively small, but in the same direction; optical rotations suggested incomplete separation of the isomers (Table I). In contrast, the *laevo* isomers, except of the ethiodide of benzhexol, were no more toxic than the *dextro* isomers, and those of procyclidine and tricyclamol were significantly less toxic ($P<0.001$).

TABLE I

COMPARISON OF (1) PERIPHERAL ATROPINE-LIKE ACTIVITIES (ESTIMATED BY MYDRIASIS IN MICE OR RELAXATION OF ACETYLCHOLINE SPASM OF GUINEA-PIG ILEUM) AND (2) INTRAVENOUS TOXICITY (IN MICE)

General formula:



X	Isomer	m.p., °C.	Rotation $[\alpha]_{5461}^{20}$ C=0·4 in $CHCl_3$	Relative Activity Atropine Sulphate=1·0		LD50† mg./kg.
				Mydriasis	Guinea-pig Ileum	
 (procyclidine)	*Racemate*	227		0·035	0·05	79
	Dextro	246	+30°	<0·003	0·002	69
	Laevo	246	−30°	0·060	0·10	86
 (tricyclamol)	*Racemate*	204–5		0·35	0·66	13 (a)
	Dextro	180	+25°	0·01	0·01	9 (a)
	Laevo	180	−25°	0·62	1·6	14 (a)
	*Racemate*	157–8		0·40	0·4	14 (a)
	Dextro	165	+30°	0·004	0·0034	12 (a)
	Laevo	165	−30°	0·76	1·0	14 (a)
 (benzhexol)	*Racemate*	241–3		0·064	0·28	60
	Dextro	264	+31°	0·025	0·075	54
	Laevo	264	−30°	0·12	0·71	57
	*Racemate*	202–3		0·46	0·57*	15*
	Dextro	183–4	+23°	0·034	0·018	12
	Laevo	183–4	−22°	1·1	0·86	14
	*Racemate*	179–180		0·32		14
	Dextro	175	+21°	0·11	Not tested	17
	Laevo	175	−18°	0·41		11
Atropine sulphate				1·0	1·0	90

* Not tested on the same occasion as the isomers. † Groups of 10 mice were used except for those estimates marked (a) – 20 mice per group.

The limits of error of the estimates of relative potency, for P=0·95, are of the order of ±20% in the mydriatic tests and ±35% in the tests on guinea-pig ileum.

Further comparison of the isomers of procyclidine and tricyclamol is presented below.

Isomers of Procyclidine

Peripheral Atropine-like Effects.—The *laevo* isomer possesses greater activity than the *dextro* by the methods referred to in Table I, and in other tests. In relaxing ACh-induced spasm of rabbit ileum, the potencies of the racemate, *dextro* isomer and *laevo* isomer were respectively 0.08, 0.0015, and 0.15 times that of atropine sulphate. In cats, under pentobarbitone sodium, the depressor effect of small doses of ACh was temporarily reduced by as little as 0.01 mg./kg. of the *laevo* isomer, and almost eliminated by 0.1 mg./kg., whereas the *dextro* isomer at doses of 2 to 5 mg./kg. only slightly reduced the effect of ACh. Vagal bradycardia was similarly affected.

Compared by their parasympatholytic effect (pressor action) in a cat infused with carbachol and adrenaline (method of Bülbring and Dawes, 1945), the activities of the racemate, *dextro* and *laevo* isomers were, respectively, approximately 0.06, 0.002, and 0.13 times that of atropine sulphate.

For protection against lethal doses of eserine (1 mg./kg. i.v.) the minimal effective doses determined in groups of ten mice were for the racemate, *dextro* isomer and *laevo* isomer, 1.5, 50, and 0.8 mg./kg. respectively.

Toxicity.—The *laevo* isomer was less toxic than the *dextro* isomer by the intravenous route in mice (Table I). Rabbits were more sensitive to both isomers; the lethal intravenous dose of each in this species was 6–10 mg./kg., and with either isomer cardiac failure preceded respiratory arrest; both isomers caused tremors and convulsions. In cats under pentobarbitone sodium, rapid falls in blood pressure (30–60 mm. Hg) occurred after 2 to 4 mg./kg. of either isomer, recovery being complete

in 2–3 min. Larger doses (10 mg./kg.) caused respiratory arrest and were tolerated only if artificial respiration was given.

Other Effects.—The isomers caused about equal inhibition of the pendular movements of the longitudinal muscle of strips of isolated rabbit ileum. In a comparative test, by adding the drugs in random order to 25 ml. baths, at the rate of 0.1 mg./min. from a mechanically driven syringe, the pendular movements of the ileum were reduced by 50% (mean of 4 trials) by the racemate at 1.2×10^{-5}, the *dextro* isomer at 1.2×10^{-5} and by the *laevo* isomer at 1.05×10^{-5}.

Little antihistamine action was shown. Thus, in relaxing histamine-induced spasm of guinea-pig ileum the activities of the racemate, *dextro* and *laevo* isomers were respectively 0.003, 0.0023, and 0.0049 times that of mepyramine. Contractions of the nictitating membrane due to pre- or postganglionic stimulation were not affected by intravenous injection of 5 mg./kg. of either isomer. Neither isomer caused analgesia or reduced the action of morphine (4 mg./kg.) when given subcutaneously at doses of 50 mg./kg. and neither at 12.5 mg./kg. intravenously reduced the duration of anaesthesia with 75 mg./kg. pentobarbitone sodium intravenously. Oxytocic action was not shown by either isomer at 10^{-6} to 10^{-5}, and the same concentrations did not prevent adrenaline (2×10^{-7}) from causing maximal contraction of isolated rabbit uterus.

Isomers of Tricyclamol

Peripheral Atropine-like Effects.—The greater anti-muscarinic action of the *laevo* isomer is shown in Table I and in other tests. Thus, in relaxing ACh-induced spasm of isolated rabbit ileum the activities of the *dextro* and *laevo* isomers were approximately 0.006 and 2 times that of atropine sulphate respectively. Further, in inhibiting the stretch reflex of isolated guinea-pig ileum the *laevo* isomer (effective at about 10^{-8}) was much more active than the *dextro* (effective at 10^{-6} to 3×10^{-6}) and about twice as potent as the racemate. Again, in cats under pentobarbitone, electrically induced vagal bradycardia was abolished and the threshold to intravenous ACh was increased by over tenfold after 0.1 mg./kg. of the *laevo* isomer, whereas more than 3 mg./kg. of the *dextro* isomer was necessary to cause similar effects. After large doses of the *laevo* isomer (2 mg./kg.) the nicotinic pressor action of ACh (500 μg.) could be observed.

Toxicity.—Respiratory arrest and paralysis of the sciatic nerve-gastrocnemius muscle preparation occurred with each isomer at doses of about 20 mg./kg. in the anaesthetized cat. Higher doses were tolerated under artificial respiration. In conscious rabbits, or in those anaesthetized with urethane, respiratory failure was again the main toxic action, occurring with either isomer or the racemic mixture at doses of 10–20 mg./kg. In mice the *dextro* isomer was slightly less toxic than the *laevo* isomer (Table I).

Other Effects.—Unlike the analogous tertiary amine (procyclidine), tricyclamol blocks the superior cervical ganglion. Like the neuromuscular effect, this action is exhibited to approximately the same extent by the *laevo* and *dextro* isomers, 5 mg./kg. intravenously causing about a 70% reduction in the contractions of the nictitating membrane due to preganglionic stimulation, without affecting those due to simultaneous postganglionic stimulation of the opposite membrane. These doses lowered the blood pressure, this action tending to be slightly greater with the *dextro* than with the *laevo* isomer.

Neither analgesia nor reduction in the analgesic effect of morphine sulphate (4 mg./kg. subcutaneously) occurred after subcutaneous injection of 50 mg./kg. of either isomer in rats, and the duration of anaesthesia due to 75 mg./kg. pentobarbitone sodium in mice was not affected by either isomer. They do not show any specific antihistamine action (guinea-pig ileum and cat blood pressure), oxytocic effect (isolated guinea-pig or rabbit uterus) or anti-adrenaline action (isolated rabbit uterus or cat blood pressure). At high concentrations (3×10^{-6}–10^{-5}) both reduced the spasmogenic action of $BaCl_2$ (5×10^{-5}) on isolated guinea-pig ileum.

Summary

1. The separation of the optical isomers of procyclidine, benzhexol, and their respective methiodides and ethiodides is described.

2. The anti-muscarinic activity of these compounds is almost entirely due to the *laevo* components.

3. The anti-muscarinic action is not related to the toxicity of the compounds, most of the *laevo* isomers being no more toxic and some (e.g. procyclidine and tricyclamol) being slightly less toxic than the *dextro* isomers.

4. In other respects the effects of the *dextro* and *laevo* isomers of the procyclidine and tricyclamol do not differ greatly ; for example, the *dextro* and *laevo* isomers of tricyclamol possess equal, though feeble, ganglion-blocking and neuromuscular-blocking actions.

The authors are indebted to Dr. D. W. Adamson and Dr. A. C. White for their interest in this work, and to Mrs. E. P. Penson and Mrs. I. A. Saunders, who carried out many of the pharmacological tests.

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Brit. J. Pharmacol. (1955), **10**, 387.

THE EFFECT OF CORTISONE ALONE AND IN COMBINATION WITH ISONIAZID IN EXPERIMENTAL CORNEAL TUBERCULOSIS IN MICE

BY

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(RECEIVED JUNE 23, 1955)

It is well known that cortisone has a striking effect in certain conditions, notably rheumatoid arthritis, and that this effect is due to its action on collagenous tissue. Indeed, it is now generally accepted that cortisone prevents the formation of new connective tissue (Ragan, Howes, Plotz, Meyer, and Blunt, 1949; Spain, Molomut, and Haber, 1950; Shapiro, Taylor, and Taubenhaus, 1951). Since such an effect would hinder the encapsulation of tubercle bacilli, and hence make them more accessible to antibiotics, many workers have studied the action of cortisone on experimental tuberculosis by a variety of methods. The results have been diverse. Thus, some investigators have found that cortisone aggravates tuberculous lesions (Hart and Rees, 1950; Bunn and Drobeck, 1952; Michael, Cummings, and Bloom, 1950) whereas others have stated that it either has no deleterious effect or even some beneficial effect (Le Maistre and Tompsett, 1951; Le Maistre, Tompsett, Muschenheim, Moore, and Dermott, 1951; Weimer, Boak, Bogen, Drusch, Miller, Moshing, and Carpenter, 1953; Greenburgh, Robson, and Willcox, 1953). Most, if not all, such investigations have been carried out on animals with intact adrenals.

In view of the discrepant results on intact animals, it seemed desirable to investigate the effect of the hormone on adrenalectomized as well as on intact animals. Since these experiments revealed striking deleterious actions, it became of interest to find how cortisone modified the effects of isoniazid, and thus a combination of the two drugs was used in both intact and adrenalectomized mice. The effects of the drugs were evaluated quantitatively by the mouse corneal method.

METHODS

All experiments were performed on mice of the albino C strain. The cornea was infected by the technique of, and with the strain of bovine tubercle bacilli used by, Rees and Robson (1950). Adrenalectomy was performed, by the dorsal approach, under ether anaesthesia one week before the corneal inoculation. The operated mice were given normal saline to drink, but this was discontinued when the hormone treatment was started. Cortisone acetate suspended in saline was injected subcutaneously, in daily doses as described in the different experiments. Isoniazid was given in M.R.C. diet 41. The daily dose in 5 g. of diet was 0.3 mg. to intact and 0.2 mg. to adrenalectomized mice, as the latter were unable to tolerate the larger dose. Treatment was started on the same day as corneal inoculation and was continued for 28 days (except in experiment 1, which lasted for 25 days), after which all surviving animals, except those treated with isoniazid alone, were killed and a post-mortem examination carried out. The isoniazid-treated animals were kept for 6–7 weeks and were then killed and examined.

To prevent secondary infection each mouse was injected daily with 2,000 units of penicillin for a few days after the corneal inoculation. However, in one experiment intensive antibiotic therapy was given: this consisted of penicillin in the same dose together with chlortetracycline (aureomycin) dissolved in the drinking-water, or saline in a dose of 100 mg./kg. body weight (on the assumption that each mouse drinks daily some 4 cc.), and was continued for the whole period of the experiment.

RESULTS

Effects of Cortisone

Two experiments were first performed to determine the effect of cortisone on corneal tuberculosis in the absence of endogenous adrenal secretion. In each experiment there were four groups of 14–18 mice: (1) intact control, (2) adrenalectomized control, (3) and (4) adrenalectomized treated with cortisone (0.1 and 1.0 mg./day respectively in Expt. 1, and 0.5 and 1.0 mg./day respectively in Expt. 2). The first experiment was stopped 25 days after inoculation, but the second was continued for a somewhat longer period and revealed important additional effects of the hormone.

Corneal lesions developed in all the inoculated eyes after the usual latent period of 10–12 days. The lesions in the control groups developed gradually, becoming dense with a caseous appearance, and were accompanied by oedema and vascularization from the limbus.

The experiments demonstrated that the course of the corneal tuberculosis was affected both by adrenalectomy and by cortisone. In both experiments the lesions developed rather more slowly in the adrenalectomized than in the control animals, though there was no qualitative difference in the course of the infection. This is illustrated in Figs. 2, 3, and 4.

Cortisone (0.5 or 1.0 mg./day) produced initially a slight beneficial effect, since it decreased the size of the corneal lesions by diminishing the amount of oedema and vascularization. Occasionally it also caused a slight increase in the latent period. At about the third week after inoculation mice treated with cortisone developed a typical corneal bleb (Fig. 1). The bleb first appeared in the centre of the caseous lesion as a transparent spot, which grew and became herniated and filled with transparent fluid. Ultimately perforation led to complete destruction of the eye, with the formation of a caseous mass. Hence with the appearance of the corneal bleb the expected course of the corneal lesion was completely changed and progressed instead in a fulminating manner (Fig. 2). This phenomenon was seen in most of the mice which survived more than some three weeks (Table I).

FIG. 1.—Enlarged photograph of mouse eye, showing developing bleb (marked with arrow) in an animal treated with cortisone, 0.5 mg./day for 21 days after inoculation.

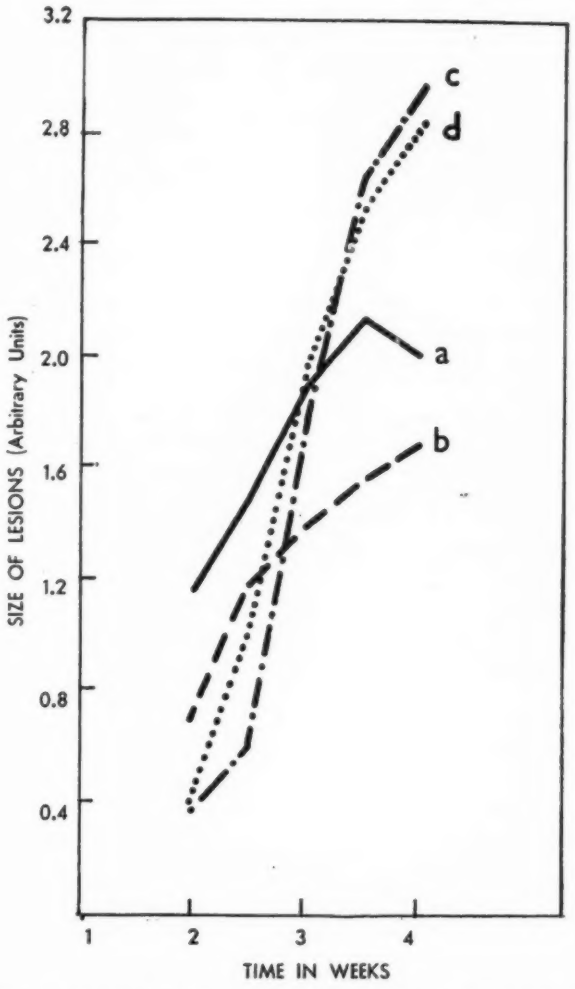

FIG. 2.—Showing the effect of the adrenal gland and of cortisone on the course of corneal tuberculosis. Average size of lesions (arbitrary units) in: (a) intact controls, (b) adrenalectomized controls, (c) adrenalectomized and treated with cortisone (1.0 mg./day), and (d) adrenalectomized and treated with cortisone (0.5 mg./day).

Cortisone in a dose of 0.1 mg./day to adrenalectomized mice had no noteworthy effect, as the lesions developed and progressed in the same manner as in the control and were accompanied by the usual oedema and vascularization.

Cortisone not only had a harmful effect in aggravating the corneal lesions after the initial stage but also led to the development of generalized tuberculosis. This was found in all the mice

TABLE I

EFFECT OF CORTISONE ON DEVELOPMENT OF BLEBS AND ON SUBSEQUENT DESTRUCTION OF THE EYES, IN MICE INOCULATED INTRACORNEALLY WITH *MYCO. TUBERCULOSIS*

Note that 0·1 mg./day had no effect, whereas 0·5 or 1 mg./day produced a striking effect

Expt.	No. of Treated Mice	Dose of Cortisone (mg.)	Mice Developing Blebs	Mice with Eyes Destroyed	Remarks
1	14	0·1	0	0	—
	16	1·0	10	1	Experiment terminated at 25 days
2	14	0·5	14	11	Three mice died before their eyes were destroyed*
	14	1·0	10	6	3 mice died before 21 days—one did not develop a bleb

* One mouse died on 20th day, a second died on 24th day, and the third was killed at the end of the experiment.

which were autopsied—five mice of the group receiving 1.0 mg. cortisone in the first experiment, and 21 mice of the two groups treated with the hormone in the second experiment. Obvious macroscopic lesions were seen in many organs, including the liver, spleen, kidneys, heart, and serous membranes, in 15 out of the 26 animals; in the remaining 11 animals there was no obvious macroscopic tuberculosis, yet microscopic examination of smears from the liver and spleen revealed the presence of enormous numbers of tubercle bacilli.

The animals lost weight, their skin lost its sheen, they became debilitated and emaciated and looked generally ill. Moreover, the dorsal adrenalectomy wound did not show signs of healing during the period of the experiment.

Effects of Isoniazid together with Cortisone

Having obtained these results with cortisone, investigations were performed to determine how cortisone modified the response to isoniazid both in intact and in adrenalectomized animals. It has been shown (Goulding and Robson, 1952) that isoniazid produces a strikingly beneficial effect on the course of corneal tuberculosis in non-adrenalectomized mice.

Two experiments were performed. In each there were 8 groups of 13–18 mice: (1) intact control, (2) adrenalectomized control, (3) intact treated with cortisone (0.5 mg./day), (4) adrenalectomized treated with cortisone (0.5 mg./day), (5) intact treated with isoniazid (0.3 mg./day), (6) adrenalectomized treated with isoniazid (0.2 mg./day), (7) intact treated with cortisone (0.5 mg./day) and isoniazid (0.3 mg./day), and (8) adrenalectomized treated with cortisone (0.5 mg./day) and isoniazid (0.2 mg./day). The isoniazid and cortisone were given for the usual period of 28 days.

In the first experiment the mice received the usual antibiotic treatment of daily penicillin. The effects of combining cortisone with isoniazid were, however, so striking—in that the infection had become more severe—that it was considered desirable to take all possible precautions to exclude secondary infection. The second experiment was therefore a repetition of the first, except that the mice received intensive antibiotic therapy, consisting of penicillin and chlortetracycline, as described under Methods.

The results of these experiments, in so far as they concern the cornea, are recorded in Figs. 3 and 4. They confirm previous results: that adrenalectomy leads to a somewhat lesser development of the tuberculous infection of the cornea and

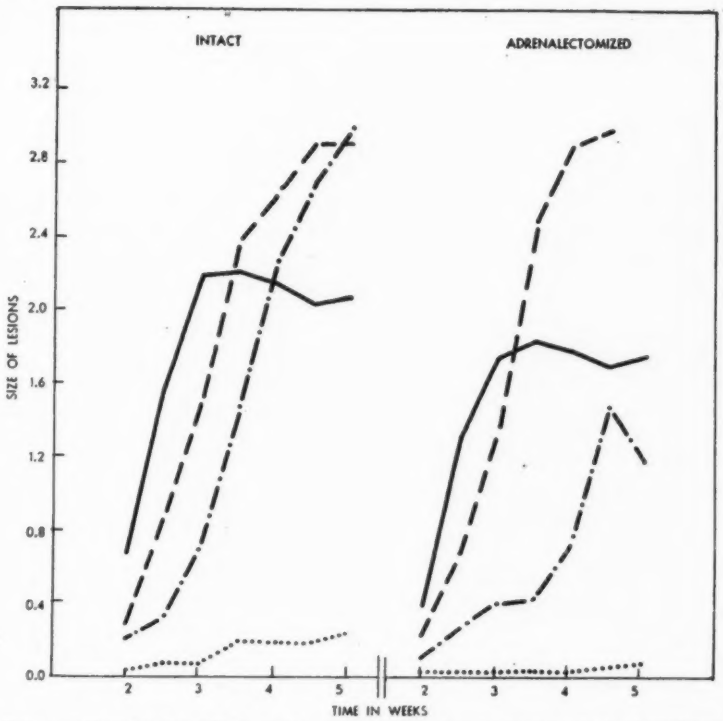

FIG. 3.—Showing the effect on the development of corneal tuberculosis of cortisone and isoniazid, in intact and adrenalectomized animals (Expt. 3). Average size of lesions in: controls (——), animals treated with isoniazid (. . . .), animals treated with cortisone (– – –) and animals treated with cortisone and isoniazid (_ . _ .).

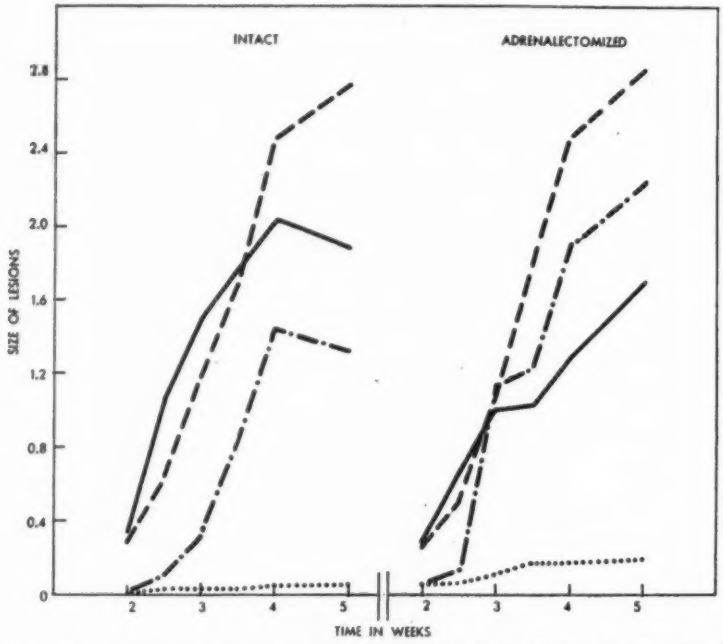

FIG. 4.—Experiment similar to that of Fig. 3, except that all animals received intense antibiotic treatment with penicillin and chlortetracycline (Expt. 4). Control (——), isoniazid (. . . .), cortisone (– – –), isoniazid plus cortisone (_ . _ .).

that the administration of cortisone to adrenalectomized mice markedly aggravates the infection after the first three weeks during which there is a slight beneficial effect. They also show that cortisone has a similar biphasic effect in animals with intact adrenal glands. These findings are remarkably similar in both experiments showing that the intensive antibiotic therapy did not appreciably diminish the deleterious action of cortisone (Table II).

TABLE II

EFFECT OF CORTISONE ALONE OR IN COMBINATION WITH ISONIAZID ON THE DEVELOPMENT OF BLEBS AND SUBSEQUENT DESTRUCTION OF THE EYES, IN MICE INOCULATED INTRACORNEALLY WITH *MYCO. TUBERCULOSIS*

Isoniazid did not prevent the deleterious effect of cortisone

Expt.	No. of Treated Mice*	Treatment	Mice Developing Blebs	Mice with Eyes Destroyed	Remarks
3	36	Cortisone	29	26	7 died before 21 days
	35	Cortisone and isoniazid	16	8	10 died before 21 days and 11 did not develop blebs
4	27	Cortisone	23	18	2 died before 21 days and 2 did not develop blebs
	28	Cortisone and isoniazid	16	3	5 died before 21 days and 7 did not develop blebs

* Both intact and adrenalectomized.

This effect was also striking in mice on isoniazid therapy (Fig. 4 and Table II). Both in intact and in adrenalectomized mice the combination of cortisone with isoniazid greatly diminished the efficacy of the tuberculostatic drug. This is seen in Expts. 3 and 4, though it is slightly less marked in the latter with its intensive antibiotic therapy. Even so, out of 28 normal or adrenalectomized mice on isoniazid and cortisone, 16 developed blebs, which led to complete destruction of the eye in 3, whereas in Expt. 3, out of 35 animals with no intensive antibiotic therapy, there were 16 blebs and 8 destructions of the eye.

The effect of cortisone in overcoming the beneficial action of isoniazid was also seen when the systemic spread of the disease was investigated (Table III). In many of the animals on combined treatment, both in Expts. 3 and 4, multiple tuberculous lesions, mainly involving the liver and spleen, were found; microscopic examination of such lesions showed many tubercle bacilli. In other animals, though no macroscopic lesions were found, smears from the liver and spleen revealed large numbers of acid-fast bacilli. It should be emphasized that in the absence of cortisone treatment we have consistently failed to find systemic spread, either macroscopically or microscopically, in many animals examined for this purpose.

Histological examination of the corneas of the various groups was performed in the hope of finding why the course of the disease was altered by the administration of the hormone. There was, however, no difference in the fibrous tissue formation or in the cellular invasion in the various groups. The corneal stroma was thickened in all the animals at the site of the lesion. In the cortisone-treated mice there was diffusion of fluid from the invading capillary blood vessels (Fig. 5). This fluid started forming in a part of the cornea adjacent to the posterior surface, gradually accumulated in an anterior direction, and led to the development of the corneal bleb with ultimate perforation. One clear result, for which there is at present no explanation in histological terms, is that the lesions in animals treated with cortisone, and some treated with cortisone and isoniazid, were teeming with

TABLE III

EFFECT OF CORTISONE ALONE OR IN COMBINATION WITH ISONIAZID ON THE DEVELOPMENT OF SYSTEMIC TUBERCULOSIS IN MICE INOCULATED INTRACORNEALLY WITH *MYCO. TUBERCULOSIS*

Note the striking development of systemic tuberculosis in the cortisone-treated mice, which was not prevented by the concomitant administration of isoniazid

Treatment	Experiment 3				Experiment 4			
	No. of Mice Treated (i)	Mice with Generalized T.B. (ii)	Mice with No Systemic Spread	No Autopsy	No. of Mice Treated (i)	Mice with Generalized T.B. (ii)	Mice with No Systemic Spread	No Autopsy
Cortisone	36	29 (3)	4	3	27	20 (3)	2	5
Cortisone and isoniazid ..	35	20 (3)	6	9	28	14 (4)	11	3

(i) Both intact and adrenalectomized. (ii) No. with microscopic lesions only in parentheses.

tubercle bacilli (Fig. 6) in contrast to the fewer organisms seen in lesions from the other groups. Nevertheless, no secondary infection was detected in Gram-stained preparations from the corneal lesions of the cortisone-treated mice.

DISCUSSION

These experiments have shown consistently that adrenalectomy produces a small, but definite, favourable effect on the course of corneal tuberculosis in the mouse. The mechanism of this action is at present unknown. It cannot be attributed to the cortisone naturally secreted by the animal's adrenals since, at this stage of the disease, cortisone produces a beneficial effect.

The effect of cortisone administration, both in intact and in adrenalectomized mice, can be divided into two stages. In the first, lasting for about three weeks, there is a beneficial effect which is probably due to the anti-inflammatory action of cortisone which is known to inhibit markedly inflammation and vascularization of the cornea as well as of other parts of the body (Jones and Meyer, 1950; Michaelson, 1952; Harvey, Howard, and Kattus, 1950; Moon and Tershakovec, 1952; Ebert, 1952).

This transient beneficial effect of cortisone is followed by a deleterious action which starts with the formation of a corneal bleb at the centre of the lesion and may ultimately result in perforation of the eye and its destruction by caseation; there is also widespread dissemination of tubercle bacilli throughout the body. These findings are in general agreement with those of Hart and Rees (1950) and other investigators, who state that cortisone exacerbates tuberculosis and produces widespread infection. It is obvious that at this stage cortisone markedly reduces resistance to the infection. Since

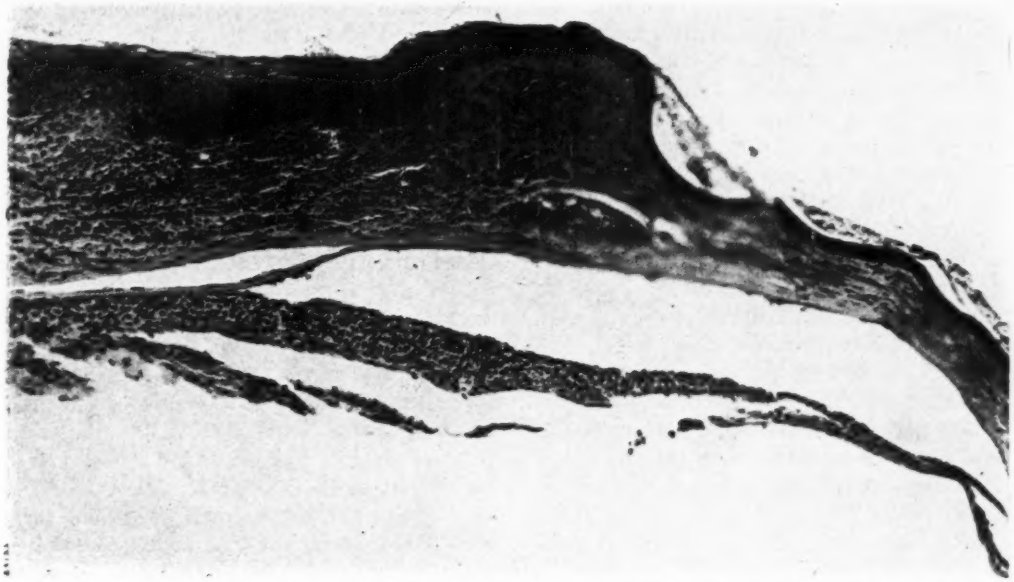

FIG. 5.—Section of cornea, showing the development of bleb. Note space in posterior part of cornea and, anterior to this, exudation of fluid from capillaries, ×100.

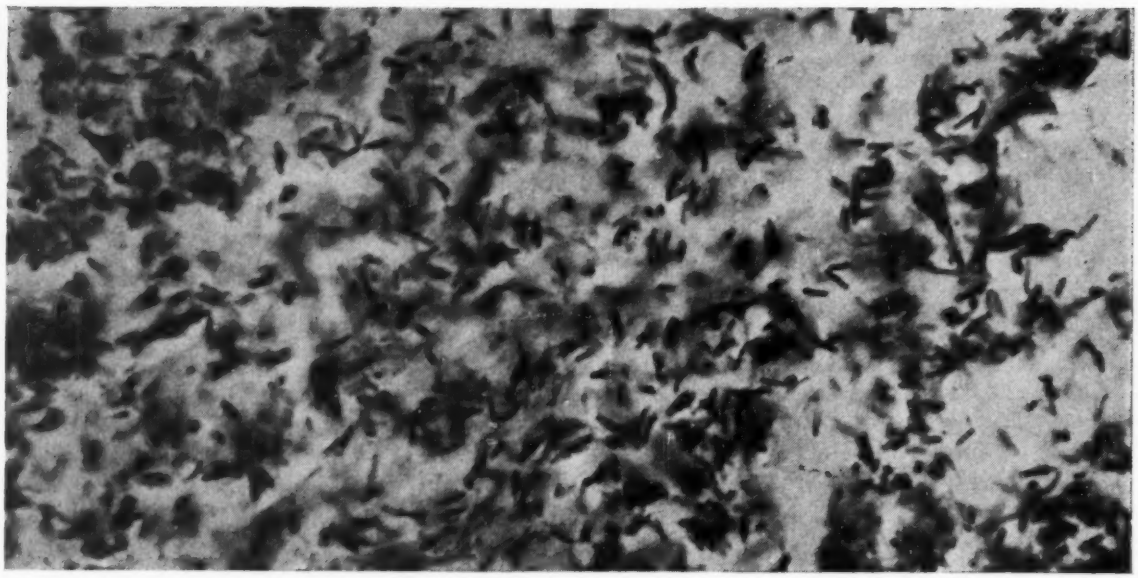

FIG. 6.—Section of the same cornea as in Fig. 5 stained with Ziehl-Neelsen to show the large number of tubercle bacilli. Animals treated with cortisone for 26 days, × 1,100.

cortisone renders animals more susceptible to spontaneous and experimentally induced pyogenic infection (Selye, 1951 ; Thomas, 1953), special precautions were taken to determine whether this effect was not, in part at least, due to secondary infection rather than to a true exacerbation of the tuberculosis. But the action of cortisone remained even when intensive antibiotic therapy was given, and, moreover, histological examination of the material failed to reveal secondary infecting organisms. It must, therefore, be concluded that cortisone truly does lead to a worsening of the tuberculous infection of the eye, as well as to the dissemination of the disease to other parts of the body. It is of interest, in this connexion, that Bunn and Drobeck (1952) found that corticotrophin, and to a lesser extent cortisone, produced a deleterious effect on ocular tuberculosis in the rabbit when the treatment was started at the time of, or shortly after, inoculation of the anterior chamber: the course of the disease was speeded, more rapid destruction of the eye occurred, and metastatic tuberculosis very often developed. In the present experiment this effect of cortisone was accompanied by a very rapid multiplication of the mycobacteria which were present in the cornea in enormous numbers at the time of bleb formation. It is possible, indeed, that the presence of these large numbers of organisms may have contributed, by their toxic effect, to the formation of the bleb and the subsequent perforation of the cornea. At present the course of these lesions is being followed in the unstained cornea by means of the phase-contrast microscope, and the presence of enormous numbers of tubercle bacilli, in cords and in palisades, has been confirmed at the stage preceding the development of the bleb. It is hoped to discuss these findings in detail in a later paper.

The results of combined treatment with cortisone and isoniazid are particularly interesting since they show that cortisone can antagonize the beneficial effect of the tuberculostatic substance, both in intact and in adrenalectomized mice, and lead to caseous destruction of the eye and the development of generalized tuberculosis. This is contrary to the findings of Ilavsky and Foley (1954), who investigated the course of tuberculosis in mice injected intraperitoneally, and found that the effect of isoniazid was not impaired by the concomitant administration of cortisone. It must be noted, however, that though the dose of cortisone used was similar, these workers used appreciably larger amounts of isoniazid than we did (about 0.8 mg./mouse/day as compared with 0.2–0.3 mg./mouse/day), and this may account for the discrepancy. Ilavsky and Foley did find that the effectiveness of streptomycin was impaired by cortisone. The mechanism by which the adrenal steroid antagonizes the strikingly beneficial effect of isoniazid in corneal tuberculosis requires further investigation.

Summary

1. The effect of adrenalectomy and of cortisone on tuberculosis was studied by the corneal method in mice.

2. Adrenalectomy produces a small, but definite, favourable effect on the course of the disease.

3. Administration of cortisone (0.5 or 1 mg./day) to intact or adrenalectomized mice produces a slight beneficial effect, which lasts for about three weeks. This is followed by a deleterious action involving the development of a corneal bleb and leading to perforation of the eye with extensive caseous involvement. Systemic spread of the disease to many organs also occurs.

4. The hormone also antagonizes the beneficial effect of isoniazid both in intact and in adrenalectomized mice; there is exacerbation of the localized tuberculosis and also metastatic infection.

5. Histological investigation of the material has not explained the mechanism of action of cortisone, though it has shown that it involves intense multiplication of the organisms.

The expenses of this investigation were in part defrayed by a grant from the Medical Research Council (to J. M. R.). Technical assistance was provided by a grant from Guy's Hospital Endowment Fund.

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THE TREATMENT OF EXPERIMENTAL BACILLARY INFECTIONS OF MICE WITH QUINOXALINE 1:4 DI-*N*-OXIDE

BY

C. E. COULTHARD AND L. J. HALE

WITH AN ADDENDUM BY M. R. GURD

From the Research Department, Boots Pure Drug Co., Ltd., Nottingham

(RECEIVED MAY 13, 1955)

Quinoxaline 1:4 di-*N*-oxide (RD 2579) was first described by McIlwain (1943), who drew attention to its bacteriostatic action against *Streptococcus haemolyticus* and *Corynebacterium diphtheriae* in *in vitro* tests. Later Frisk (1946) showed that it possessed tuberculostatic activity, and more recently Jones, Landquist, and Stewart (1953) reported amoebicidal properties, and made incidental reference to its activity against Gram-negative bacteria. Hurst, Landquist, Melvin, Peters, Senior, Silk, and Stacey (1953) found it effective in the therapy of experimental psittacosis and lymphogranuloma venereum (inguinale).

A sample of RD 2579 was supplied by Dr. W. F. Short in August, 1952. We were impressed by its action against Gram-negative bacteria, and particularly by the fact that not only was it bacteriostatic for *Salmonellae*, *Shigellae*, and coliforms, but also for each of thirteen strains of *Pseudomonas pyocyanea* and of eleven strains of *Proteus vulgaris* against which it was tested. This activity was maintained virtually undiminished in blood-containing media.

METHODS

In vitro *Tests.*—Dilutions in papain digest broth showed that RD 2579 inhibits growth of Gram-negative bacteria at concentrations of about 0.01 to 0.003%.

In vivo–In vitro *Tests.*—The antibacterial activity of RD 2579 in mice was investigated by a modification of the method described by Croshaw and Foley (1954).

The drug was administered to groups of mice and these were sacrificed at intervals. The blood was removed by heart puncture, pooled, and allowed to clot; the separated serum was then diluted in two-fold steps in papain digest broth in test tubes. The tubes were sown with one drop of a diluted 24 hr. broth culture of the test organism and incubated at 37° C. in parallel with dilutions in the same broth of normal mouse serum sown in the same way.

In vivo *Tests.*—A number of tests were carried out to investigate the protection afforded by RD 2579 in experimental infection of mice by *Salmonella typhi*, *Salmonella paratyphi B*, *Shigella sonnei*, or *Pseudomonas pyocyanea*.

Albino mice were used throughout and in any one experiment all were of the same sex and strain and, as far as possible, of the same weight. This was usually 25–30 g.

RESULTS

Table I shows typical results of a number of *in vivo–in vitro* tests against a variety of Gram-negative bacteria. The serum of treated mice remained bacteriostatic for at least 2 hr. after an intraperitoneal dose of RD 2579.

TABLE I

IN VIVO–IN VITRO ACTIVITY OF RD 2579 (0.32 MG./G. INTRAPERITONEALLY) AGAINST GRAM-NEGATIVE BACTERIA

Test Bacterium	Time after Administration during which the Serum Remained Bacteriostatic (hr.)	Highest Dilution of Serum at which Activity was Detected
Sh. sonnei	3	× 4
,, ,, *	2 or longer	× 2
Salm. typhi	,, ,, ,,	× 4
Ps. pyocyanea (mixed inoculum of 13 strains) ..	,, ,, ,,	× 8
Prot. vulgaris (mixed inoculum of 11 strains) ..	,, ,, ,,	× 16

* Drug given orally in this test.

Table II shows the effect of the drug upon the survival time of infected mice. In the last experiment in Table II, all local lesions in treated animals cleared in 24–72 hr. At the end of the experiment a pure culture of *Ps. pyocyanea* was obtained from the lesion on the surviving control animal.

This last experiment was modified in accordance with suggestions made by the late Professor F. R. Selbie, who had, at our suggestion, carried

TABLE II

MOUSE PROTECTION TESTS AGAINST VARIOUS GRAM-NEGATIVE BACTERIA

(Intraperitoneal inoculation of organisms in 5% mucin)

Infecting Organism	Dose of RD 2579 and Mode of Administration	Treated Survivors and Duration of Experiment	Untreated Survivors and Time of Survival
Salm. paratyphi B	2×0·13 mg./g. i.p. at 0 and 24 hr. after infection	4/5 6 days	0/5 6 days
Salm. paratyphi B	1×0·32 mg./g. orally 5½ hr. after infection	10/10 8 days	3/10 8 days
Salm. typhi	1×0·26 mg./g. orally 2 hr. after infection	10/10 4 days	0/10 24 hr.
Sh. sonnei ..	1×0·2 mg./g. orally 2 hr. after infection	9/10 5 days	0/10 24 hr.
Ps. pyocyanea	1×0·32 mg./g. orally 2 hr. after infection followed by 1×0·16 mg./g. 4 hr. later	5/10 3 days	1/10 3 days
*Ps. pyocyanea**	3×0·125 mg./g. i.m. right thigh at 2, 24, and 48 hr. after infection	8/10 14 days	1/10 14 days
	3×0·25 mg./g. i.m. right thigh at 2, 24, and 48 hr. after infection	10/10 14 days	
	1×0·5 mg./g. i.m. right thigh at 2 hr. after infection	5/10 14 days	

i.p., intraperitoneal injection. i.m., intramuscular injection.
In the "survivors" column the fractions indicate $\frac{\text{survivors}}{\text{number at risk}}$.

* In this test the organisms were injected i.m., in broth, to left thigh.

out a number of experiments with RD 2579 against infections in mice of strains of *Ps. pyocyanea*. In a personal communication Professor Selbie pointed out that RD 2579 not only prevented or delayed death but that it caused marked diminution and ultimate disappearance of local lesions deliberately produced by this technique.

Details of three of Professor Selbie's experiments with three strains of *Ps. pyocyanea* are as follows:

On the basis of *in vitro* bacteriostatic titres of RD 2579 and streptomycin with strains "YDE," "Kilner" and "Sinclair," protection tests were carried out in mice. Eighteen mice were used in each test, and each was infected with 0.2 ml. of a 1 in 5 dilution of an 18 hr. broth culture by injection in the left thigh muscle. Two hours after infection six mice were given RD 2579 (250 mg./kg.) subcutaneously, six received streptomycin (125 mg./kg.) also subcutaneously, and six remained untreated. The size of the local lesion was measured in each survivor 24 hr. after infection by deducting the width of the normal thigh from that of the affected thigh.

Table III shows the results obtained in these tests by Professor Selbie.

TABLE III

COMPARISON BETWEEN RD 2579 (250 MG./KG.) AND STREPTOMYCIN (125 MG./KG.) GIVEN SUBCUTANEOUSLY TO MICE INFECTED WITH STRAINS OF *PS. PYOCYANEA*

Test	Strain of Bacteria	Treatment	Width of Lesions in mm. 24 hr. after Infection in Mouse No.:					
			1	2	3	4	5	6
1	YDE ..	None	D	D	D	D	D	D
		RD 2579	6·0	4·5	4·5	4·0	3·0	1·5
		Streptomycin	D	D	D	D	8·0	7·5
2	Kilner ..	None	7·5	7·0	7·0	6·5	6·5	5·0
		RD 2579	2·0	2·0	1·5	1·0	1·0	1·0
		Streptomycin	7·5	6·5	6·5	6·0	5·5	5·5
3	Sinclair ..	None	6·5	6·0	5·0	4·5	3·5	2·5
		RD 2579	1·5	0·5	0·5	0·5	0·5	0·0
		Streptomycin	1·5	1·5	1·0	1·0	0·5	0·5

Width of lesion is the difference in mm. between normal and affected thighs.
D, mouse died.

Compatibility with Antibiotics.—A 0.1% aqueous solution of RD 2579 does not suffer appreciable alteration in its antibacterial effect when penicillin, aureomycin, chloramphenicol or oxytetracycline are dissolved in it at a concentration of 0.05% and incubated at 37° C. for 24 hr. The activity of the antibiotics also appears to remain relatively unchanged in the mixture. Agar dilution experiments with strains of *Ps. pyocyanea* and *Prot. vulgaris* have shown that combination of the compound with streptomycin delays the development of resistance to the latter, and there would appear to be a synergistic action which is most marked with strains of *Prot. vulgaris*.

SUMMARY

1. Quinoxaline 1:4 di-*N*-oxide, whether administered orally or by injection, protected mice against experimental infection with *Salmonella typhi, S. paratyphi B, Shigella sonnei,* and *Pseudomonas pyocyanea.*

2. It appears to delay the development of resistance of strains of *Ps. pyocyanea* and *Prot. vulgaris* to streptomycin, and to be synergistic with the latter.

3. It was effective *in vitro* against all strains of *Prot. vulgaris* investigated, but since none of these was pathogenic we have been unable to carry out *in vivo* experiments.

The authors thank Dr. W. F. Short for supplies of the compound, Mr. G. W. Inkley for practical help and advice, and Mr. W. Metcalf for skilled technical assistance.

ADDENDUM

Toxicity of RD 2579

By M. R. Gurd

The main toxic effect of RD 2579 given systemically is on the liver and kidneys (Jones *et al.*, 1953). In our rats, repeated oral doses of 200 mg./kg. daily caused severe cellular degeneration in the liver and less severe damage to the kidneys. Daily oral doses of 50 mg./kg. caused mild liver damage, without discernible damage to the kidneys. When administered subcutaneously mild toxic effects such as inhibition of growth were noted with doses as low as 6 mg./kg. daily.

The compound had little or no irritant effect when injected intrathecally or into the anterior chamber of the eye. The toxicity to leucocytes was also low.

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Printed in Great Britain by Fisher, Knight & Co., Ltd., Gainsborough Press, St. Albans, Herts.